Muscle regeneration in the soft palate of the rat

Novel perspectives for cleft palate repair

Paola Liliana Carvajal Monroy

Colofon

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In memory of

† Aldemar Carvajal Arias
-My father-

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Chapter 1

Introduction

"The bigger difficulty

was the speech. Hugo's palate was operated on just after his first birthday. He became an immensely chatty toddler. From the age of about one and a half, he developed an obsession with cars, … The first one he learned was Mini, which he could say more or less perfectly. But others were less readily comprehensible. BMW was a strange concoction of vowels and glottal stops, unrecognizable to all but his parents; Honda was "Onna"; Porsche was "Orrs... So just before he was three, Hugo had a third operation, to elongate his palate; and then, six months later, he had another, more minor one. For the past two years, he has had regular speech therapy…"

William Sckidelsky. The Guardian, 2015

The clinic has been the greatest source of inspiration to propose and conduct this research project. In October 2015, an article about the experience of a parent having a child with cleft lip and palate was published in the British newspaper 'The Guardian' (see quote text left page). In this article, I clearly recognize many problems that I encounter daily. Therefore, I will use this story to take you along the introduction of this thesis.

Cleft lip and/or palate (CL/P) is the most common congenital facial malformation in humans. Every three minutes a child with an orofacial cleft (deformity) is born, especially in developing countries this constitutes a major problem where millions of children are suffering from untreated clefts.[1] In the Netherlands, the incidence of CL/P is 1.6 in 1.000,[2] while, in Colombia, it varies from 1 in 500 to 1 in 1.000 births.[1] CL/P can be associated with other anomalies and may be part of a syndrome. Clefts are generally divided into two groups based on both genetic and embryological grounds; clefts involving the anterior structures (lip and primary palate) and clefts involving only the soft palate or extending into the hard palate (secondary palate).[3-5] Hence CL/P is a highly heterogeneous group of disorders affecting the upper lip, the nose, and the oral cavity (Figure 1).[6] A complete description of abnormal and abnormal development of primary and secondary palates has been published recently.[7] In this publication, a new classification for orofacial clefts based on both cell biological mechanisms (e.g. fusion and differentiation defects), and time in embryonic development is proposed. This new method enables more accurate determination of etiology through gene identification and/or environmental interactions.[7,8]

The treatment of these patients is complex, and involves a multidisciplinary team and includes multiple interventions (e.g. surgical repair, speech therapy, hearing control/management and orthodontic treatment). It starts before birth (prenatal counseling) and lasts until early adulthood (Figure 2).[9]

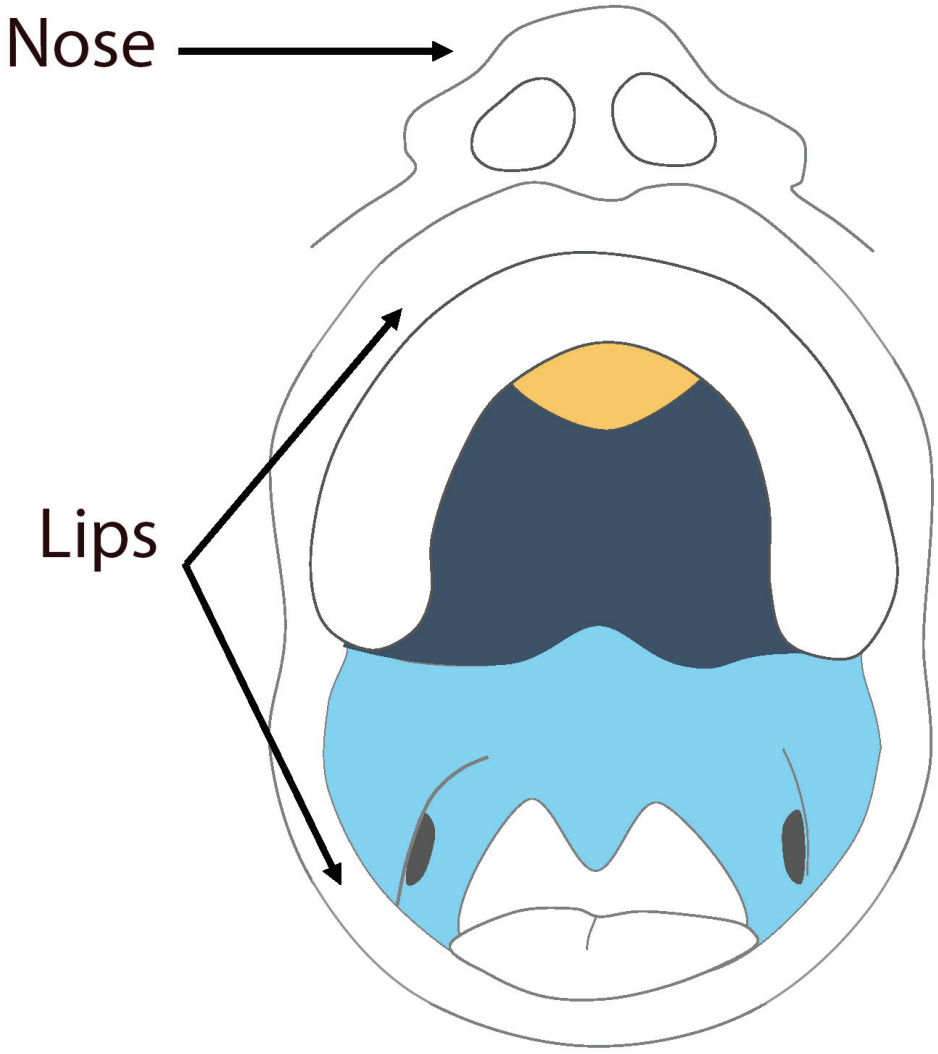

Figure 1. Schematic representation of the oral cavity. The palate separates the nasal from the oral cavity. The primary palate (yellow), and secondary palate (blue). The secondary palate is divided into a hard bony palate (dark blue) and a muscular soft palate (light blue).

Figure 2. Unilateral cleft lip and palate. The treatment is complex and multiples interventions (e.g. surgical repair, speech therapy, and orthodontic treatment) are required. It starts before birth (prenatal counseling) and lasts until early adulthood. Adapted from: www.schisisonderzoek.nl

From the bed …

The palate separates the nasal from the oral cavity. It is divided into an anterior bony (hard) palate and a posterior muscular (soft) palate (Figure 1). The levator veli palatini (LVP) is the major muscle of the soft palate and occupies about 40% of the total length of the soft palate.[10] It elevates the soft palate and extends posteriorly sealing off the pharynx. The LVP is thus crucial for the normal functioning of the soft palate during speech, blowing, swallowing and sucking. In non-cleft subjects, the LVP originates bilaterally at the skull base (petrous portion of the temporal bone) and fans out into the soft palate to join its counterpart at the midline. In contrast, the fibers of the LVP in patients with cleft lip and palate insert into the bony cleft edges and/or posterior edge of the hard palate (Figure 3).

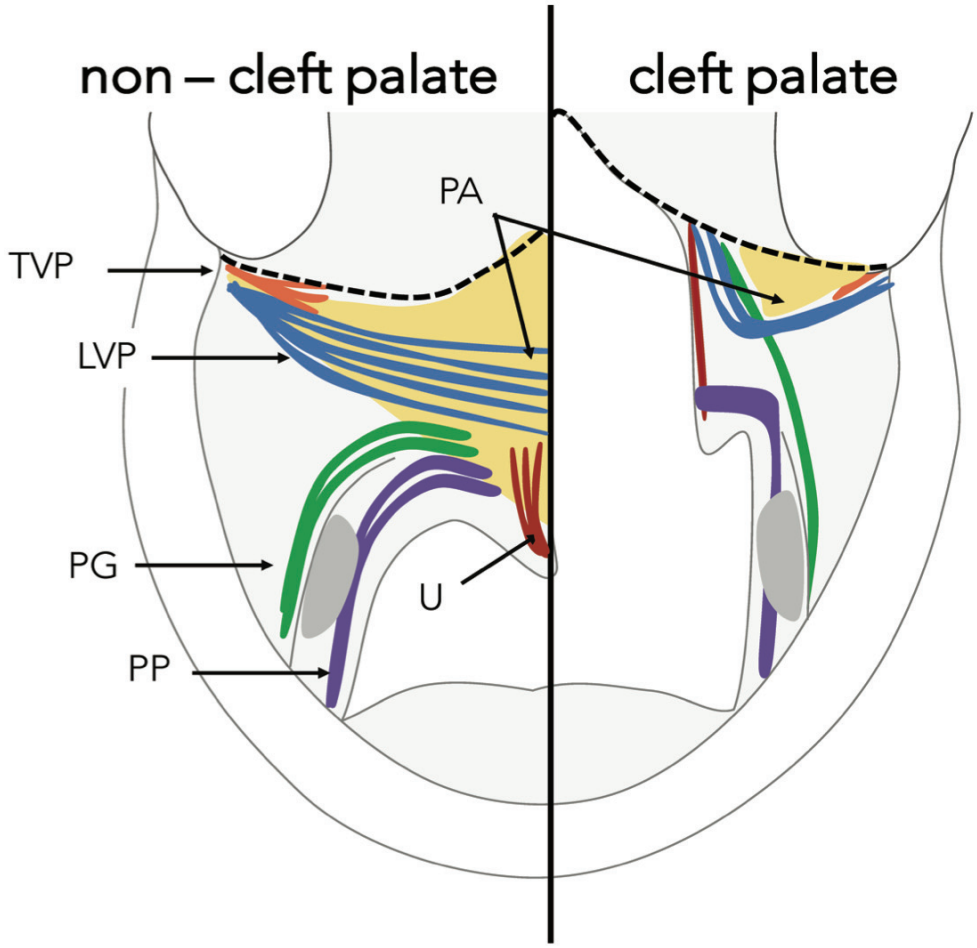

Figure 3. Muscles of the soft palate. m. tensor veli palatini (TVP), m. levator veli palatini (LVP), m. palatoglossus (PG), m. palatopharyngeus (PP), m. uvulae (U), and palatal aponeurosis (PA). The dashed line indicates the posterior border of the hard palate. Notice the abnormal insertion of the soft palate muscles into the posterior border of the hard palate at the cleft side.

Only after attention was raised for the abnormal insertion of palatal muscles, new surgical techniques were developed with the aim to reconstruct the levator muscle sling.[10-12] Nowadays, muscle reconstruction is considered the most critical component of the surgical repair of the soft palate.[10] Although there is a wide range of surgical protocols, soft palate repair generally takes place in early childhood[13] and aims at optimal function of the velopharyngeal sphincter and speech development.[14,15]

The main morbidity associated with a cleft in the soft palate is velopharyngeal dysfunction (VPD). This is the general term used to describe the inability of the soft palate to achieve complete velopharyngeal closure (Figure 4). The velopharyngeal sphincter is composed of the soft palate anteriorly, the lateral pharyngeal walls, and the posterior pharyngeal wall. Velopharyngeal closure is accomplished by the combination of the movement of all these structures. The velopharyngeal sphincter should remain open for normal breathing and for the production of nasal consonants (/m/, /n/, and /ng/). In contrast, complete closure of the velopharyngeal sphincter is required for the production of oral pressure consonants (e.g. /p/, /b/, /t/, /d/, /k/, /g/, /s/, /z/, /f/, /v/), vowels /a/, /e/, /i/, /o/ /u/), sucking, swallowing and blowing (Figure 4).[13] Various patterns of velopharyngeal closure have been described.[16]

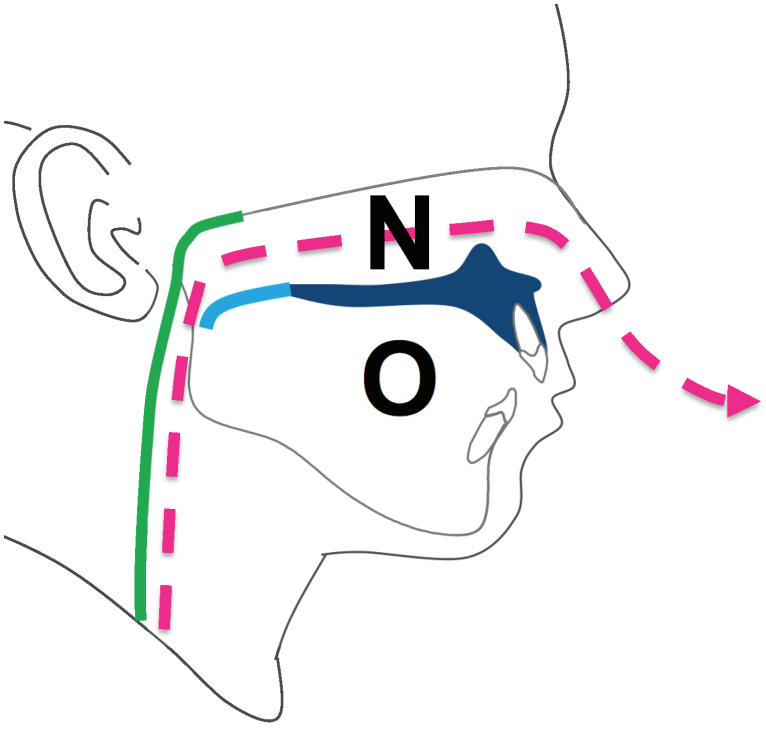

Figure 4. Lateral view of the airway. N: nasal cavity, O: oral cavity, green: posterior pharyngeal wall, dark blue: hard palate, light blue: soft palate, and pink: air escapes through the nose when consonants such as "p", "b", "g", "t", and "d" are pronounced. This phenomenon is called velopharyngeal dysfunction.

Despite the wide range of surgical techniques for soft palate repair[17], about 10 to 30% of the patients is still not able to achieve complete velopharyngeal closure after surgery.[18,19] This results in hypernasal speech (primary effect of VPD) and other functional deficits such as nasal escape and articulation disorders that often require a second surgical correction in 25% of these patients.[18,19] Furthermore, the child may develop compensatory speech patterns (secondary effects of VPD), which can affect his social interaction with other children and adults. In fact, more adolescents with an operated cleft express concerns about their speech than about their facial appearance.[20]

VPD can be diagnosed both by subjective and objective means. Perceptual evaluation of speech and nasometry by an experienced speech therapist remains the standard.[21,22] In addition, intraoral inspection, videofluoroscopy and nasoendoscopy provide visual information of velopharyngeal closure. Speech therapy is thus necessary to correct articulation errors while surgery is required to correct nasal air escape and hypernasality. The type of surgical procedure depends on the preoperative pattern of velopharyngeal closure.[23] Surgical interventions include posterior pharyngeal wall augmentation, redirection of the anteriorly displaced LVP, palatal lengthening, sphincter pharyngoplasty and pharyngoplasty.[24]

Other problems commonly associated with the surgical closure of the soft palate are the formation of oronasal fistula. Oronasal fistula have been reported in about 8% of cases when only surgical closure of the soft palate is performed.[15] Fistula can also lead to air escape contributing to the symptoms of hypernasal speech.[25] Facial growth seems not to be affected by closure of the soft palate only.[26] When a cleft involves both the soft and the hard palate a one - or two - stages protocol is used: in the one-stage protocol both hard and soft palate are closed at the same time. The side effects of this protocol are assumed to include disturbances of facial growth. The two-stage protocol consists of

two separate operations. Soft palate and hard palate are closed at different time points. The closure of the hard palate can be delayed until the phase of mixed dentition, so around 8-9 years old. The claimed advantage of this second technique is that it may avoid gross disturbances of facial development. However, a lot of controversy remains on the issue of optimal timing. Hence, randomized prospective clinical trials are warranted to determine the optimal timing of palatoplasty with respect to long-term speech development and maxillofacial growth.[27]

… to biological side

Deficiencies in speech remain frustratingly common in children with a history of a cleft in the soft palate. This can have a devastating effect on their social interaction with other children and adults.[20] Several factors may contribute to suboptimal repair and hampered speech, such as age at the moment of surgery, skills and experience of the surgeon, type and extension of the cleft, scar tissue formation, damage of the motor nerve supply of the LVP muscle, the muscle fiber type distribution, and impaired muscle regeneration.[19,28-37] From all these factors, two biological aspects drew our attention: first, impaired muscle regeneration and second, scar tissue formation in the muscle layer (fibrosis).

Skeletal muscles possess a remarkable ability to regenerate after injury. The progenitor cells that are responsible for muscle regeneration are the satellite cells (SCs).[38,39] After injury, SCs undergo proliferation, differentiation and fusion to form new myofibers or repair damaged ones.[40,41] Although muscle repair is an important component of contemporary palatoplasty, little is known about muscle regeneration of the soft palate muscles after cleft palate repair. In the presence of excessive collagen accumulation, injured muscles heal slowly and often show incomplete functional recovery.[42] Eventually, suture repair of the

muscle sling can favour healing, but even this does not prevent fibrosis.[43] Scar tissue formation in the palatal mucosa has already called attention, because of its detrimental effects on maxillary growth.[44-47] However, the influence of scar tissue on the repaired soft palate and on muscle regeneration cannot be underestimated since it is probably responsible for impaired muscle function after surgical repair of the clefted muscles.

In the last two decades, several strategies based on tissue engineering have been employed to improve muscle regeneration. Growth factors, satellite cells, biological and synthetic scaffolds or a combination of these have been applied with varying results.[48-56] Most studies on muscle regeneration, however, have been performed on limb muscles, while studies on head muscles are scarce. It is important to understand head muscle regeneration since accumulating evidence suggests that muscles from limb and head muscles differ in several aspects. Skeletal muscles from the trunk and limbs are, for example, derived from the somites,[57] while most head muscles including those of the soft palate are derived from the branchial arches.[58-60] Freeze or crush injuries of the head muscles heal much slower than similar injuries in limb muscles.[61] Moreover, more fibrous connective tissue is formed during healing. In addition, SCs from head muscles also express a distinct profile of transcription factors including unique factors such as Tcf21.[62] All this indicates that the physiology of SCs from limb and head muscles is different, and that they react different to injury.[63] Thus, soft palate muscles may possess a less effective regenerative response after injury, which may be a critical factor in cleft healing after surgery.[64]

In summary, both impaired muscle regeneration and scar formation (fibrosis) hamper the functional recovery of the soft palate muscles after cleft palate repair. Novel therapies based on tissue engineering that prevent scar formation and stimulate muscle regeneration may improve the functional recovery after

surgical repair. These therapies will offer new perspectives for the treatment of patients with cleft lip and palate.

Let's start!

To date, adjuvant therapies based on tissue engineering for soft palate repair are not available, and a basis for proper translation is missing. Therefore, the general aim of this research project was to gain knowledge in the understanding of the biology and regeneration of branchiomeric–derived muscles. We started with a literature review on myogenesis in the non-cleft and cleft palate, the characteristics of soft palate muscles, and the process of muscle regeneration, which is reported in chapter 2. Also, novel therapeutic strategies based on tissue engineering to improve soft palate function after surgical repair are proposed. These adjuvant therapies are expected to improve the outcome after surgical repair by targeting new aspects of muscle healing which were not considered before.

As mentioned earlier, little is known about muscle regeneration after surgery. Therefore, a newly *in vivo* model for the study of muscle regeneration in the soft palate of rats is described in chapter 3. Subsequently, we investigated the long-term regeneration of the soft palate muscles and the formation of fibrotic tissue after excisional injury in chapter 4.

In vitro culture systems that allow the study of SCs from head muscles are crucial to develop new therapies based on tissue engineering. Hence, in chapter 5, we presented a new protocol for the isolation, culture and differentiation of SCs from rat head muscles. In chapter 6 we investigated the differences between branchiomeric head muscles and limb muscles in rats of two ages, and the properties of the SCs. Lastly, principal findings are summarized and discussed according to their current scientific context in the general discussion presented in chapter 7.

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CHAPTER 2

STRATEGIES TO IMPROVE REGENERATION OF THE SOFT PALATE MUSCLES AFTER CLEFT PALATE REPAIR

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Tissue Eng Part B Rev. 2012; 18 (6): 468-77

Abstract

Children with a cleft in the soft palate have difficulties with speech, swallowing, and sucking. These patients are unable to separate the nasal from the oral cavity leading to air loss during speech. Although surgical repair ameliorates soft palate function by joining the clefted muscles of the soft palate, optimal function is often not achieved. The regeneration of muscles in the soft palate after surgery is hampered because of (1) their low intrinsic regenerative capacity, (2) the muscle properties related to clefting, and (3) the development of fibrosis. Adjuvant strategies based on tissue engineering may improve the outcome after surgery by approaching these specific issues. Therefore, this review will discuss myogenesis in the non-cleft and cleft palate, the characteristics of soft palate muscles, and the process of muscle regeneration. Finally, novel therapeutic strategies based on tissue engineering to improve soft palate function after surgical repair are presented.

Cleft lip and/or palate (CLP) is the most common congenital facial malformation in humans. It occurs in about 1/500 to 1/1000 births, with ethnic and geographic variation.[1] This disorder is generally divided into two groups: clefts involving the lip with or without cleft palate and isolated cleft palate[2] (Figure 1). CLP can also be part of a syndrome when it is associated with other congenital defects.[3] In this review we will focus on clefts involving the soft palate.

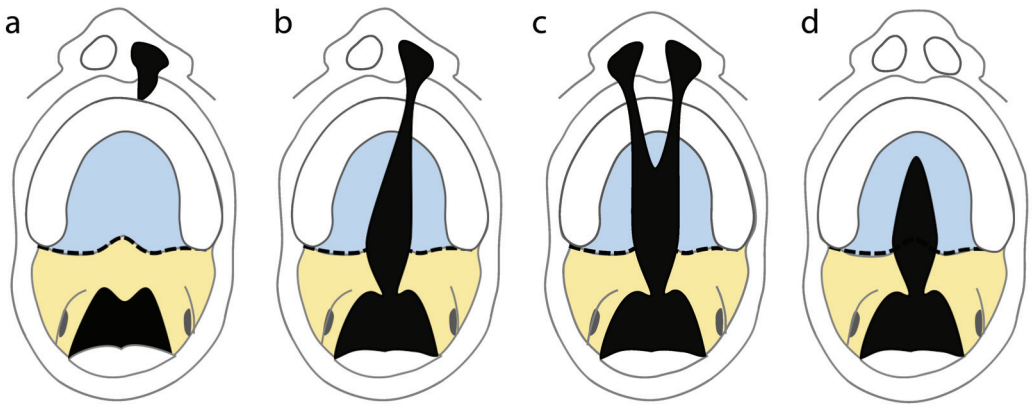

Figure 1. Cleft lip and/or palate. (a) Cleft lip. (b) Unilateral cleft lip and palate. (c) Bilateral cleft lip and palate. (d) Isolated cleft palate. The dashed line indicates the posterior border of the hard palate.

Children with a cleft in the soft palate have difficulties with speech, swallowing, and sucking. These patients are unable to separate the nasal from the oral cavity; a phenomenon known as velopharyngeal dysfunction. Surgical repair is required to close the defect and to reconstruct the muscle sling of the m. levator veli palatini (LVP), the major muscle of the soft palate[4] (Figure 2). It ensures optimal function and normal speech development.[5,6] However, about 10% to 30% of the treated patients remain unable to achieve adequate velopharyngeal function.[7,8] This results in

hypernasal speech that often requires additional surgical corrections. Diverse factors are attributed to suboptimal repair such as age at the time of the surgery, skills and experience of the surgeon, type and extension of the cleft, fibrosis, damage of the motor and proprioceptive innervation, and abnormal fiber type distribution.[7,9–12]

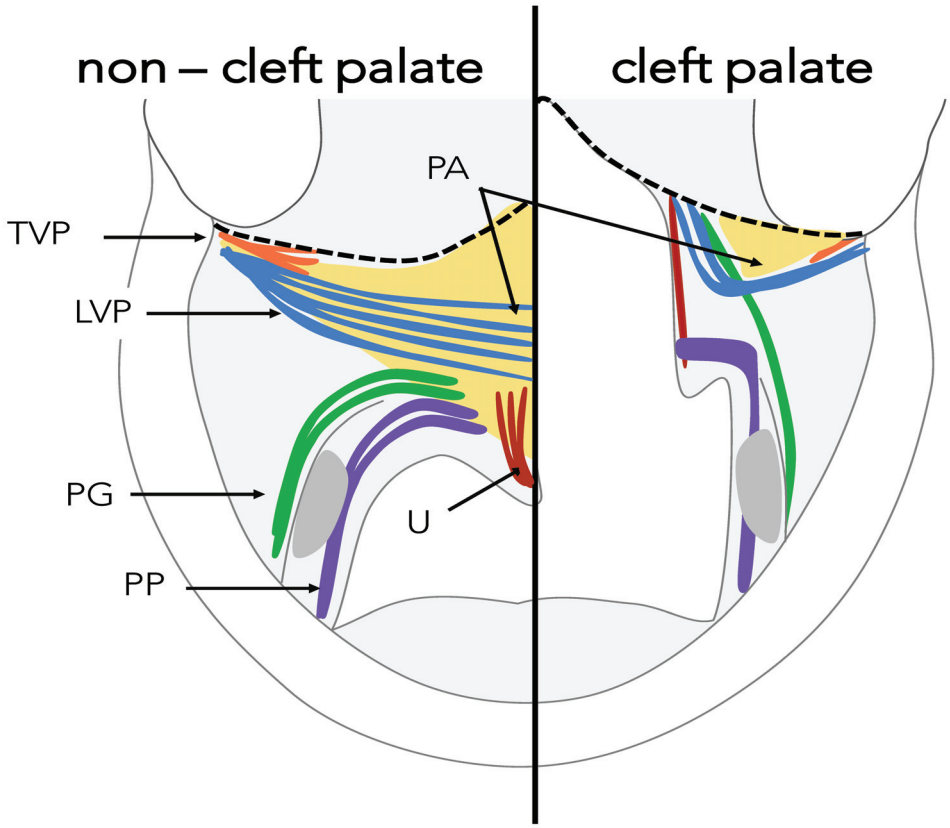

Figure 2. Muscles of the soft palate. m. tensor veli palatini (TVP), m. levator veli palatini (LVP), the major muscle of the soft palate, m. palatoglossus (PG), m. palatopharyngeus (PP), m. uvulae (U), and palatal aponeurosis (PA). The dashed line indicates the posterior border of the hard palate. Notice the abnormal insertion of the soft palate muscles into the posterior border of the hard palate at the cleft side. In this area disorganized myofibers are present. Cleft palate muscles are limited to isometric contractions and often underdeveloped.

This review will highlight the myogenesis and characteristics of the soft palate muscles in the non-cleft and cleft palate, and the process of muscle regeneration. We postulate that muscle healing and function after soft palate repair can be improved by adjuvant

strategies from the field of regenerative medicine. How these novel adjuvant strategies based on tissue engineering could improve muscle healing and prevent velopharyngeal dysfunction after surgical repair will be discussed.

Soft palate myogenesis

The muscles of the soft palate and those controlling jaw movement, facial expression, and pharyngeal (throat) and laryngeal (voice box) function are known as branchiomeric muscles.[13,14] The formation of the soft palate muscles starts already early in embryonic development and yields five pairs of muscles: (1) the tensor veli palatini (TVP), (2) the LVP, (3) the palatopharyngeus (PP), (4) the palatoglossus (PG), and 5) the uvulae (U) (Figure 2). Mesenchymal condensations of the individual soft palate muscles emerge sequentially, starting from the sixth week of gestation in humans, with the TVP appearing first and the U last. Their full morphological development is completed after the 17th week of intrauterine life. The hard palate is already fused at this stage. The masticatory muscles and the TVP develop at the same time, and are followed by the morphogenesis of the LVP, PP, PG, and U muscles, and by the palatal aponeurosis (PA).[15,16] The development of the soft palate muscles in cleft patients is delayed compared with non-cleft individuals.[17]

Soft palate muscles in the normal and cleft palate

The soft palate is formed by an interweaving of muscles from the skull base (TVP and LVP), tongue (PG), and pharynx (PP; Figure 2). All muscles extend from nearby bony structures and are inserted into the PA located in the center of the soft palate (Figure 2). Hence, all muscles have only one skeletal insertion, with the

exception of the U, which is a completely intrinsic muscle of the soft palate without bony attachment. In the cleft palate, the muscles are attached to the posterior border of the hard palate (Figure 2). The abnormal insertions of the muscles, in particular the LVP, in cleft palate patients prevent normal functioning. These muscles have two instead of one skeletal attachments limiting them to isometric contractions.[18] As a result, the cleft muscles pull the two halves of the soft palate in a superior and lateral direction, causing further widening of the cleft.[19] In addition, the LVP may become severely atrophic because of reduced function, and often has only half of the thickness found in healthy newborns. The LVP myofibers are also disorganized next to the cleft margin.[16,17]

Both slow and fast fibers are present in the soft palate muscles. Slow fibers are highly resistant to fatigue, with a low activation threshold, whereas fast fibers are more fatigable, with a higher activation threshold. In non-cleft individuals, the LVP contains predominantly slow fibers,[20,21] whereas in cleft individuals it contains a higher proportion of fast fibers and a reduced capillary supply compared with normal LVP muscles.[22–24] A fetal myosin heavy chain isoform is also present in a small number of fibers in all palatal muscles. In comparison with limb muscles, the individual and intramuscular variability in fiber diameter of the LVP is much larger.[21] The higher number of fast fibers in cleft patients may cause the increased fatigability of the LVP during speech,[23] which contributes to velopharyngeal dysfunction.[25] Further, fast fibers are more prone to contraction-induced injury.[24,26,27]

Muscle regeneration in the soft palate

After injury. Satellite cells (SCs)[28] are the primary muscle stem cells, and are responsible for postnatal muscle growth, maintenance, and repair. After injury, adjacent SCs become activated and

migrate to the site of injury, proliferate, differentiate, and fuse to form new myofibers or repair damaged ones.[29,30] In addition, signaling molecules from infiltrating macrophages, injured myofibers, and the disrupted extracellular matrix participate in the regeneration process.[29–31] The formation of scar tissue may prevent proper muscle regeneration.[32–34] The majority of studies on muscle regeneration have been performed in limb muscles, while studies on head muscles are scarce. Muscles from the limbs and the head differ in several aspects. Skeletal muscles from the trunk and limbs are derived from the somites[35] during embryonic development, while most head muscles including those of the soft palate are derived from the branchial arches.[36–38] The masseter muscle, a branchiomeric muscle, seems to contain less SCs than limb muscle.[39] Freeze or crush injuries in the masseter muscle also regenerate much slower than similar injuries in limb muscles.[40] Moreover, much more fibrous connective tissue is formed in the damaged area. Proliferating SCs from limb and head muscle also express a distinct profile of transcription factors.[39] *In vitro*, SC-derived myoblasts from masseter proliferate more but differentiate later than those from limb muscle. Remarkably, both SC populations are similarly able to regenerate limb muscle injuries after transplantation.[39] These observations support the crucial role of the SC niche in muscle regeneration.

As mentioned, fast fibers are predominantly found in cleft palate muscles compared with normal soft palate muscles.[22–24] In general, the percentage of SCs in fast muscle fibers is significantly lower than in slow muscle fibers.[41,42] This may further decrease the SC number in cleft palate muscles. In addition, *in vitro* studies show that SCs from fast muscle fibers proliferate less than those from slow muscle fibers.[39] In rodent models for muscle atrophy induced by disuse, SCs decrease in number and display reduced function.[43–45] Further, impaired macrophage recruitment to the site of injury and impaired macrophage function has been recently

demonstrated in this muscle atrophy model.[46] A similar situation may exist in cleft palate muscles that are limited to isometric contractions and are therefore also atrophied. The accompanying poor capillary supply may further impair the regeneration process and promote fibrosis.[18,19]

Disorganized muscle fibers are found at the attachment of cleft palate muscles to the posterior border of the palatal bone.[16,17] This possibly prevents further postnatal myofiber maturation by normal function.[47]

In summary, the lower regenerative capacity of branchiomeric muscles in general, and the specific properties of cleft palate muscles may compromise muscle regeneration following surgical repair in cleft patients.

Soft palate muscles in animal models

Several mammalian species have been used for the study of the normal and clefted palate. The most frequently used animal models are goats, mice, and rats.[48–53] Up to now, tissue engineering studies in the soft palate have not been performed. Therefore, the selection of an appropriate animal model for the study of the regeneration of the soft palate muscles is highly important. The use of an existing congenital cleft palate model in the goat[51,54–56] is not feasible due to the high costs that limits group size. Cleft palate models in knockout mice are generally limited to embryological studies due to high mortality after birth, and lack of reproducibility of the induced clefts.[57–60] However, the widespread use of rodents for fundamental and preclinical research ensures the availability of extensive tools and markers. In addition, their low cost and ease of handling make them the most suitable model for the development of soft palate muscle engineering techniques.

Similar to humans, the soft palate muscles in rodents possess a mixture of slow and fast fibers,[61] and play an important role in swallowing and respiration.[61,62] In addition, the soft palate muscles in cleft animals also run parallel to the margins of the cleft, and disorganized myofibers are present.[60] In contrast to humans,[17] the development of the palatal muscles in cleft palate mice is not delayed.[63]

The advantage of the rat over the mouse is its larger body size favoring surgical manipulation of the soft palate. This makes the rat the most suitable animal for experimental research on muscle regeneration of the soft palate.

Tissue engineering to improve the outcome of surgery

As mentioned in the previous sections, several biological factors have been attributed to functional impairment of the soft palate after surgical correction (Figure 3): (1) Low intrinsic regenerative capacity of soft palate muscles. As shown, branchiomeric muscles including those of the soft palate, have a low number of SCs with a low rate of differentiation compared with limb muscles, which may contribute to their poor regenerative capacity. (2) Muscle properties related to clefting. Clefted soft palate muscles contain a lower number of myofibers and are highly disorganized close to the cleft margin. This impedes the function of the reconstructed muscle. (3) The development of fibrosis. The main harmful effect of surgery is the development of muscle fibrosis, which impairs the regeneration of muscle fibers. Strategies based on tissue engineering may ameliorate the functional outcome of surgical repair by approaching these three main issues.

Another factor that may influence the regeneration of palatal muscles is age. In general, the number of SCs in growing postnatal muscle is about 30%–35% of all nuclei.[64–66] In adulthood, only 1–4% of all nuclei belong to the SC population.[67] Surgical repair of the

soft palate generally takes place in early childhood (6–36 months of age). After birth, the soft palate muscles are still immature. A large number of SCs are present to fulfill the demands of growth.[68] In contrast, newborn SCs seem to engraft less efficiently than their adult counterparts.[69] This should be taken into account when developing tissue engineering strategies for soft palate muscles.

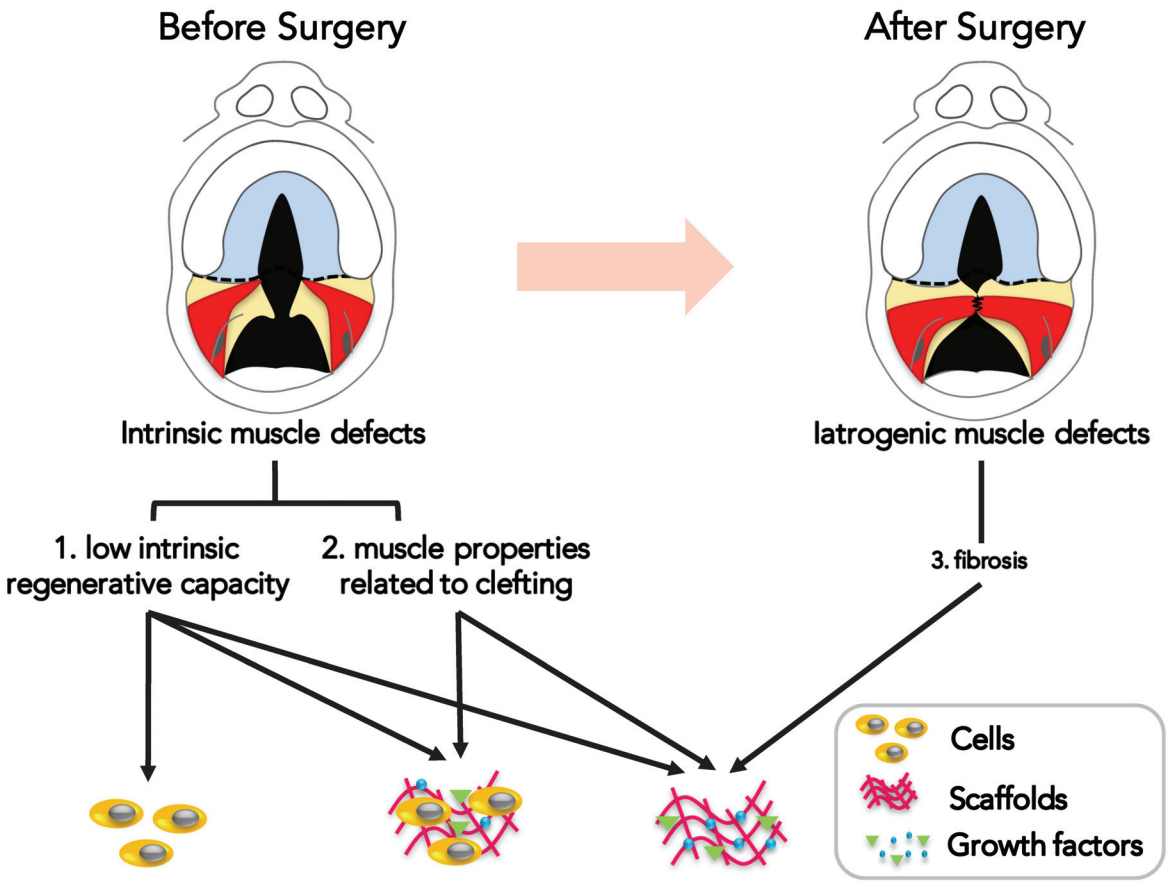

Figure 3. Muscle problems related to clefting. Before surgery: (1) Low intrinsic regenerative capacity: less number of satellite cells with a low rate of differentiation, (2) Muscle properties related to clefting: clefted soft palate muscles contain lesser number of myofibers and are highly disorganized. After surgery: (3) Fibrosis. Adjuvant strategies based on tissue engineering using (stem) cells, growth factors, and scaffolds may improve the outcome after surgery.

Improving the intrinsic regenerative properties of the soft palate muscles

Isolated SCs may be used for cell-based therapy to improve the regenerative capacity of the soft palate muscles. It has been demonstrated that transplanted SCs are able to form new myofibers and to self–renew.[70] This approach has already been used for the treatment of a variety of diseases such as muscular dystrophy[71] and myocardial infarction.[72–74] Unfortunately, the success of transplantation of SCs and myoblasts has only been limited. After isolation, SCs seem to lose their regenerative capacity in culture. In addition, their survival and migration to the site of injury after injection is limited.[75,76]

Mesenchymal and embryonic stem cells can also differentiate into skeletal muscle.[77–79] However, only a small fraction of cultured mesenchymal stem cells undergo fusion into myofibers[80] suggesting that these cells may not be an appropriate cell type for therapeutic use.[81] Embryonic stem cells have a large potential for cell-based therapies for a wide range of diseases.[82,83] These cells may offer additional advantages such as their larger replicative potential, and the possibility to perform gene targeting.[84] However, embryonic stem cell-derived myogenic populations with possible therapeutic value have not been found up to now.

Alternatively, other non-muscle stem cells such as mesoangioblasts and pericytes also have myogenic potential.[85,86] Both mesoangioblasts and pericytes can be delivered systemically because of their ability to cross the endothelium. This approach was successful for cell therapy in muscular dystrophy.[86,87] Nevertheless, the exact ability of non-muscle stem cells for muscle regeneration and their possible use in cell therapy remain to be elucidated.[88–91]

As an alternative approach, the recruitment, proliferation, and differentiation of SCs already present in the soft palate muscle may be stimulated by specific growth factors. As mentioned, muscle

growth and regeneration is controlled by locally-produced growth factors, which stimulate SCs such as insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 2 (FGF-2). IGF-1 favors muscle regeneration by increasing the rate of SC proliferation and the formation of myotubes.[92–96] Similarly, FGF-2 stimulates muscle regeneration *in vitro* and *in vivo* by promoting the proliferation of myoblasts after injury.[97–100] Moreover, it facilitates the recruitment and proliferation of SCs[101] and promotes angiogenesis.[102,103] However, when growth factors are injected *in vivo*, they rapidly lose their biological activity due to diffusion and/or enzymatic inactivation.[104] Because of this, growth factors and attachment motifs have been incorporated into biodegradable scaffolds for controlled release.[105] With microspheres prepared from materials such as polylactic-co-glycolic acid, the release of such factors can be controlled.[106–108] In addition, fibrin and gelatin hydrogels can give a sustained release of (growth) factors.[109–112] These new techniques may improve the delivery of factors to the site of injury and stimulate muscle regeneration.

Although cell-based therapy seems to be promising for the treatment of genetic muscle defects, it is probably not a feasible option for cleft palate because it is a completely different type of condition. For example, in degenerative diseases like Duchenne muscular dystrophy, multiple cycles of muscle degeneration and regeneration deplete the SC population.[113] In contrast, in cleft palate, muscle SCs are preserved. In summary, growth factors and cytokines in a suitable delivery system may stimulate proliferation and differentiation of resident SCs in the soft palate muscles after surgical repair.

Correcting cleft-associated muscle abnormalities

Muscle is a highly organized structure consisting of long, parallel multinucleated myotubes to generate sufficient force for contraction.[114] Therefore, the random myofiber orientation in cleft

palate muscles impedes normal function after surgical repair. The reduced function limits the switch from a fast to a slow myofiber type.[12,24] This may explain the increased muscle fatigability observed in patients with velopharyngeal dysfunction.[23]

Proper alignment of regenerating myotubes may be achieved with scaffolds of specific design and surface topography.[115] Myoblasts are known to respond to the surrounding topography,[116] which leads to directional cell growth.[115] Hence, different techniques have been described to control cellular alignment on micro- and nanostructured surfaces produced either by chemical or topographic patterning. These include electrospinning,[117] photolithography,[118] and electron beam lithography.[119]

Aligned nanofiber meshes can be fabricated from biocompatible polymers such as poly-lactide or poly-glycolideco lactide via electrospinning.[119] Muscle cells cultured in these meshes show alignment and migration and a contractile phenotype.[120,121] However, up to now the possibilities to control the mechanical properties of these polymer meshes is limited.[120] In addition, slowly degrading polymers persist in the regenerated tissue for a long time. Since the repair of the soft palate is generally performed in children, later growth of the soft palate must be accommodated. Therefore, the use of rapidly degrading polymers seems to be more suitable. Recently, it was reported that growth factors can be printed onto sub-micron polystyrene fibers. [122,123] A combination of a suitable scaffold with growth factors and cytokines (e.g., stromal cell derived factor–1α) might favor migration,[124] differentiation of resident SCs, and induce myofiber alignment.

Particularly in patients with wider clefts, the amount of soft palate tissue is limited, which causes tension at the junction of the hard and soft palate after surgical repair. It has been proposed that scarring affects the length of the soft palate in anteroposterior direction,[125] which depends on the specific technique used for

closure.[126] Alternatively, scarring may contribute to a narrowing of the residual cleft after two stage palatoplasty.[126–128] The site most likely to fistulize is at the junction of the hard and soft palate.[129] Fistulae usually have been associated with the type of cleft, technique and timing of repair, and the experience of the surgeon.[130] Substitutes as decellularized dermal allograft have been successfully used to close wide defects while preventing fistulae formation.[131] The use of scaffolds in the soft palate muscles allows a defined architecture to guide cell growth and development.

Preventing secondary effects of surgery: fibrosis

The deposition of excess ECM during regeneration may lead to fibrosis and impaired muscle function. A key factor in fibrosis is transforming growth factor-beta 1 (TGF-β1). [132,133] It stimulates the synthesis of collagen and other extracellular matrix components and promotes myofibroblast formation.[134] Myostatin, another member of the TGF-β family, inhibits muscle regeneration by the inhibition of SC and myoblast proliferation.[135–138] In this way, TGF-β1 and myostatin synergistically amplify the fibrotic process after injury. In contrast, myostatin knockout mice develop less fibrosis and display improved skeletal muscle regeneration compared with wild-type mice.[139]

The inhibition of TGF-β1 and myostatin may prevent fibrous scar formation and improve muscle healing after injury.[139] Decorin is a member of the small leucine-rich proteoglycan family, and it reduces both TGF-β1 and myostatin activity.[139–142] It thereby enhances the proliferation and differentiation of myogenic cells,[143] and prevents fibrosis.[144] In addition, decorin upregulates the expression of a variety of myogenic markers including Myf5, Myf6, MyoD, and myogenin *in vitro*.[145] Hepatocyte growth factor increases decorin production by fibroblasts.[146] Furthermore, small

signaling molecules such as nitric oxide down regulate TGFβ1 activity.[147] However, the importance of nitric oxide in muscle regeneration must be further elucidated. In summary, the use of decorin and other factors that inhibit TGF-β1 and myostatin may prevent scar formation after surgical correction of the soft cleft palate. A disadvantage might be that anti-fibrotic therapy can diminish narrowing of the residual cleft after two-stage palatoplasty. Therefore, the long-term effects of novel therapies based on tissue engineering in such a complex anatomical environment is hard to predict. Further research should elucidate the final outcome of these new therapeutic approaches

Conclusions

This review discusses the development and characteristics of the soft palate muscles in the non-cleft and cleft palate and the process of muscle regeneration. Finally, tissue engineering strategies are proposed to improve muscle regeneration after the closure of the soft palate. The regeneration of muscles in the soft palate after surgery is hampered because of (1) their low intrinsic regenerative capacity, (2) muscle properties related to clefting, and (3) the development of fibrosis. We propose the following strategies based on tissue engineering, to improve the functional outcome after surgical repair.

To improve muscle regeneration (problem 1), growth factors and cytokines might be applied to the surgical wounds by incorporation into scaffolds or microspheres. The growth factors should enhance SC recruitment from the adjacent muscle tissue and their proliferation and differentiation into new myofibers. This will enhance the intrinsic regeneration capacity of the soft palate muscles, and thereby increase the functional outcome of surgery in terms of number and maturation of myofibers.

Suitable scaffolds may also support the alignment of myofibers and compensate the limited amount of tissue in clefted muscles (problem 2). These scaffolds should offer an architecture that guides cell growth and myofiber formation. In terms of function, this approach will increase muscle volume and force generation by the soft palate muscles.

To prevent fibrosis (problem 3), TGF-β1 and myostatin activity can be inhibited by factors such as decorin. This will reduce the formation of fibrotic tissue and stimulate proliferation of myoblasts. The function of the soft palate muscles after repair will thus improve by allowing myofiber formation and alignment (approaches 1 and 2).

Wrapping up, scaffolds that give a controlled release of growth factors, guide the alignment of new myofibers, and prevent fibrosis might improve muscle regeneration after surgical repair of the cleft soft palate. Nevertheless, further research is required in order to develop these functionalized scaffolds.

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“... sometimes

there are very simple experiments and very simple discoveries to make using what is at hand.”

Andre Geim. Nobel Prize in Physics, 2010

In vivo studies

CHAPTER 3

A RAT MODEL FOR MUSCLE REGENERATION IN THE SOFT PALATE

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Abstract

Children with a cleft in the soft palate have difficulties with speech, swallowing, and sucking. Despite successful surgical repositioning of the muscles, optimal function is often not achieved. Scar formation and defective regeneration may hamper the functional recovery of the muscles after cleft palate repair. Therefore, the aim of this study is to investigate the anatomy and histology of the soft palate in rats, and to establish an *in vivo* model for muscle regeneration after surgical injury.

Fourteen adult male Sprague Dawley rats were divided into four groups. Groups 1 (n=4) and 2 (n=2) were used to investigate the anatomy and histology of the soft palate, respectively. Group 3 (n=6) was used for surgical wounding of the soft palate, and group 4 (n=2) was used as unwounded control group. The wounds (1 mm) were evaluated by (immuno)histochemistry (AZAN staining, Pax7, MyoD, MyoG, MyHC, and ASMA) after 7 days.

The present study sho ws that the anatomy and histology of the soft palate muscles of the rat is largely comparable with that in humans. All wounds showed clinical evidence of healing after 7 days. AZAN staining demonstrated extensive collagen deposition in the wound area, and initial regeneration of muscle fibers and salivary glands. Proliferating and differentiating satellite cells were identified in the wound area by antibody staining.

This model is the first, suitable for studying muscle regeneration in the rat soft palate, and allows the development of novel adjuvant strategies to promote muscle regeneration after cleft palate surgery.

Cleft lip and/or palate (CLP) is the most common congenital facial malformation in humans. It occurs in about 1:500 to 1:1000 births, with ethnic and geographic variation.[1] CLP is generally divided into clefts involving the lip with or without cleft palate, and isolated cleft palate.[2] In 20 to 34% of the cases it is part of a syndrome, and associated with other congenital defects.[3] About 45% of all patients with CLP have a cleft of the soft palate.[4]

The levator veli palatini is the major muscle of the soft palate, which moves it up and down. This muscle is therefore critical for the functioning of the soft palate during speech, swallowing, and sucking. Children with a cleft palate can not separate the nasal from the oral cavity during speech, a phenomenon known as velopharyngeal dysfunction.[5,6] The surgical repair of the soft palate normally takes place early in childhood at 6–36 months of age, although the protocols are highly variable.[2] Surgery is required to close the defect and to reconstruct the palatal muscles.[7] The aim is to restore the function of the soft palate allowing normal speech development.[8,9] However, velopharyngeal dysfunction persists in 7 to 30% of the patients, despite anatomical repositioning of the muscles during surgery.[5,6,10–12] This results in speech abnormalities.[13] Various factors such as age at the time of the surgery, skills and experience of the surgeon, type and extension of the cleft, and damage of the motor and proprioceptive nerves have been attributed to suboptimal repair.[5,14–16]

In general, muscle tissue possesses a large ability to regenerate. Satellite cells (SCs) are the primary muscle stem cells, and responsible for postnatal muscle growth, maintenance, and repair.[17] Upon injury, SCs are activated and migrate to the wound, proliferate, differentiate, and form new myofibers or repair damaged ones.[18] SCs are located between the basal lamina and the plasma membrane,[17,19] and express the transcription factor Pax7.[20,21] A distinct gene expression profile characterizes the SC

progeny.[22,23] The myogenic determination factor 1 (MyoD) is expressed during SC proliferation, whereas differentiation is marked by a decline in Pax7 expression, and the induction of myogenin (MyoG).[24] Differentiating myoblasts express various genes that encode structural proteins such as myosin heavy chain (MyHC), and finally fuse to form myotubes.[25,26] SC differentiation and, hence, muscle repair is regulated by signaling molecules from infiltrating macrophages, injured myofibers, and the disrupted extracellular matrix.[18,27]

Several strategies have been used in regenerative medicine to improve muscle regeneration. Growth factors, satellite cells, biological and synthetic scaffolds, or a combination of these have been applied to injured muscles with varying results.[28–32] Most studies on muscle regeneration, however, have been performed in limb, trunk muscles, while studies on head muscles are scarce. Skeletal muscles from the trunk and limbs are derived from the somites during embryonic development,[33] while most head muscles, including those of the soft palate, are derived from the branchial arches.[34–36] Interestingly, head muscles generally contain less SCs than limb muscles.[37] Head muscles also regenerate much slower than limb muscles after freeze, crush or similar injuries, and more fibrous connective tissue is generally formed during healing.[38] Proliferating SCs from head muscles also express a different profile of transcription factors.[37] In addition, the muscles in the soft palate of CLP patients are smaller than normal palatal muscles and the myofibers are not properly organized.[39,40] All this may contribute to the poor regeneration of soft palate muscles after surgical closure of the soft palate.[41] Taken together, scar formation and incomplete muscle regeneration seem to be the main causes of muscle dysfunction after cleft palate repair, next to the already mentioned factors.

Up to now, no animal models are available to investigate muscle regeneration in the soft palate. Therefore, the aim of this study was

to describe the anatomy and histology of the soft palate in rats, and to establish an *in vivo* model for muscle regeneration in the soft palate after surgical injury.

Material and Methods

Animals

Ethics statement. Approval of the research protocol was obtained from the local Board for Animal Experiments (Dier Experimenten Commissie) from the Radboud University Nijmegen in accordance with Dutch laws and regulations (RU-DEC 2011-125).

Fourteen adult male Sprague Dawley rats, weighing 280–300 g (Harlan BV, Horst, The Netherlands) were housed under standard laboratory conditions. The rats had been acclimatized to the animal facility for one week, before the start of the experiments. The animals were divided into four groups, group 1 (n=4) and group 2 (n=2) to investigate the anatomy and histology of the soft palate, respectively. Group 3 (n=6) was used for surgical wounding of the soft palate, and group 4 (n=2) was used as unwounded control group.

Experimental Procedures

Dissection techniques

Anatomical dissection of the soft palate was performed in four animals (Group 1) using an operating microscope (Carl Zeiss AG, Oberkochen, Germany) after euthanasia with CO_2/O_2. In the first stage, as described elsewhere,[42] a ventral incision was made extending from the mandibular symphysis to the clavicle. The subcutaneous tissues were separated until the submandibular gland was visible. By removal of the salivary gland, the digastric and sternocleidomastoid muscles were then exposed. The posterior belly of the digastric muscle was dissected to its origin, and pulled laterally to expose the tympanic bulla. This procedure was

conducted at both sides, and the levator veli palatini was carefully dissected from its origin to its insertion in the soft palate. In the second stage, the mandibular ramus was cut on both sides, and the soft palate was dissected to examine the origin and course of the tensor veli palatini muscle.

In group 2, the animals were decapitated after sacrifice with the standard CO_2/O_2 protocol, and the heads were fixed in 4% paraformaldehyde (PFA) for 24 hours at 4°C, and then rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Serial coronal sections of 5 µm were cut for histochemical staining after decalcification in 10% ethylenediaminetetraacetic acid (EDTA) and paraffin embedding.

Excisional wounding of the soft palate

Six animals (group 3) received buprenorphine (0.02 mg/kg s.c.; Temgesic, Schering Plough, Brussels, Belgium) as analgesic before surgery, and also at the next two days with twelve hour intervals. General anesthesia was induced with a mixture of ketamine (75 mg/kg i.p.; Nimatek, Eurovet Animal Health B.V, Bladel, The Netherlands) and medetomidine (0.5 mg/kg i.p.; Dexdomitor, Janssen–Cilag B.V, Tilburg, The Netherlands). In addition, atropine (0.05 mg/kg i.m.; Atropine, Pharmachemie B.V, Haarlem, The Netherlands) was injected to prevent medetomidine-induced bradycardia. The animals were placed in a supine position on an operating table tilted at 45° to allow optimal surgical exposure of the soft palate. Body temperature was kept at 38°C using a heating pad.

All procedures were performed in semi-sterile conditions under an operating microscope (Carl Zeiss AG, Oberkochen, Germany) by the same operator (P.L.C.M) trained in microsurgical techniques. Chlorhexidine digluconate gel 0.2% (Orosol; Fresenius Kabi B.V, Schelle, Belgium) was used to clean the surgical area. Excisional wounds (1 mm ø) were made in the soft palate using a biopsy punch, seven mm behind the ninth palatal ruga (Figure 1). Reversion of the anesthesia was induced with atipamezol hydrochloride (0.5 mg/kg

i.p.; Antisedan; Janssen-Cilag B.V, Tilburg, The Netherlands). The animals received powdered chow in water during the following three days. Their behavior was monitored daily with special attention for water and food intake, loss of weight, and activity. The animals were euthanized with the standard CO_2/O_2 protocol after seven days.

Histology

After euthanasia, the soft palates of the six experimental animals (group 3) and two control animals (group 4) were dissected, fixed in 4% PFA in PBS, and processed for paraffin embedding. Paraffin sections of the two heads (group 2) and the eight soft palates (group 3 and 4) were stained with azocarmine G and aniline blue (AZAN) to discriminate collagen (blue) from muscle tissue (red).

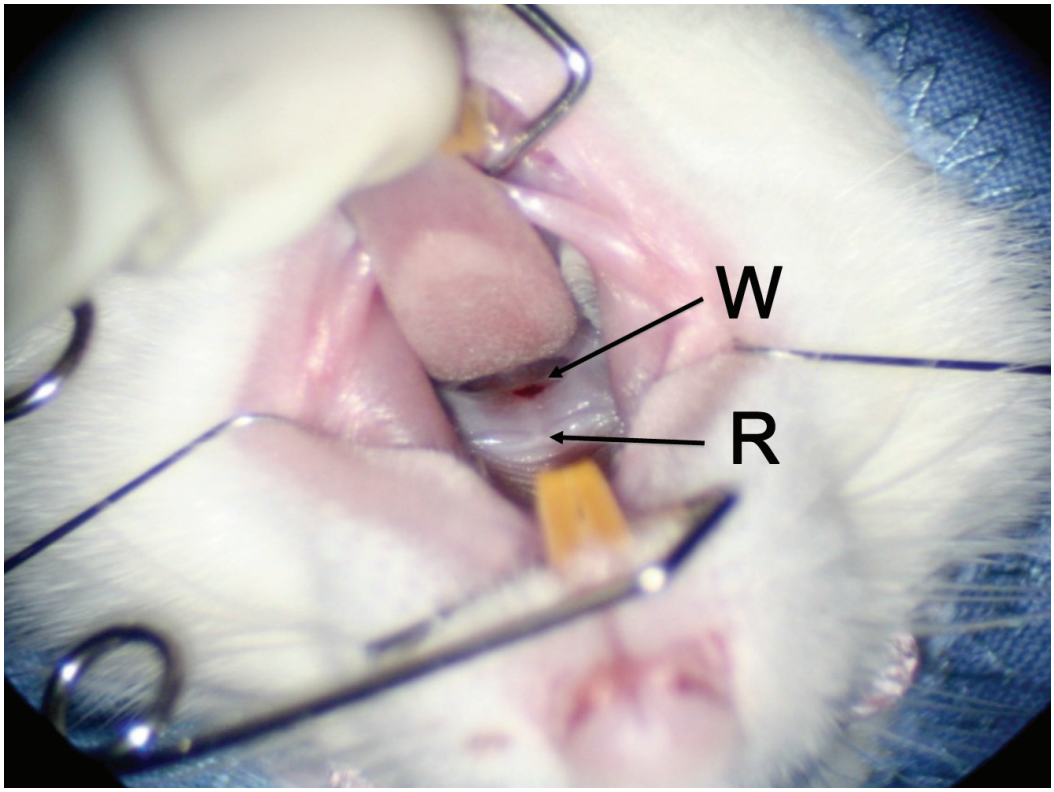

Figure 1. Excisional wounding of the soft palate. Excisional wounds (1 mm ø) were made in the soft palate, 7 mm behind the 9th palatal ruga. W: excisional wound, R: 9th ruga.

Immunohistochemistry

Sections were deparaffinated, rehydrated, treated with 3% H_2O_2 for 20 minutes to inactivate endogenous peroxidase, and post-fixed with 4% PFA in PBS. For Pax7 and MyoD staining, the sections were first heated in 0.25 mM EDTA/10 mM TRIS buffer (pH 9.0) at 100°C for 10 minutes. For MyoG staining, the sections were first heated in citrate buffer (pH 6.0) for 40 minutes at 100°C. For alpha-smooth muscle actin (ASMA) and fast myosin skeletal heavy chain (MyHC) staining, the sections were heated in citrate buffer (pH 6.0) at 70°C for 10 minutes, and subsequently treated with 0.075% trypsin in PBS (pH 7.4) for 15 minutes to retrieve antigens. Sections were then incubated with mouse anti-ASMA (1:10.000; Chemical CO, St Louis, MO, USA), mouse anti-fast MyHC (1:5000; Sigma Chemical CO, St Louis, MO, USA), mouse anti-Pax7 (1:100; Developmental Studies Hybridoma Bank, Iowa City, CA USA), mouse anti-MyoD (1:50; DAKO, Dakopatts, Glostrup, Denmark), or mouse anti-myogenin (1:100; Developmental Studies Hybridoma Bank), overnight at 4°C. Next, the sections were incubated with biotinylated secondary antibodies donkey-anti-mouse IgG (H+L) (1:500; Jackson Labs, West Grove, Pa, USA), and a preformed biotinylated horseradish peroxidase - avidin complex (Vector Laboratories, Burligame, CA, USA). The sections were stained with 3,3-diaminobenzidine substrate, and analyzed qualitatively.

Results

Anatomy of the Soft Palate Muscles

In adult rats, the length of the soft palate is about 11 mm, which is relatively longer than in humans. It extends from the posterior edge of the hard palate (9th ruga) towards the nasopharyngeal sphincter (Figure 2).

Figure 3 shows a schematic drawing of the levator veli palatini and tensor veli palatini muscles. Clinical pictures are presented in figures

4 A to D. The levator veli palatine muscle originates from the inferior surface of the temporal bone, and runs in medial and anterior direction (Figure 4A). It then runs posterior to the pterygoid process towards the soft palate, crosses the midline, and joins the contralateral levator veli palatini muscle fibers forming a muscle sling (Figure 4B). The glossopharyngeal, vagus and hypoglossal nerves are visible between the levator veli palatini and the skull base (Figure 4A and 4B). The tensor veli palatini originates from the inferior surface of the sphenoid bone, the lateral surface of the pterygoid plate, and the auditory tube (Figure 4C). It runs towards the pterygoid process and its tendon curves around the pterygoid hamulus. It then continues medially towards the soft palate and forms the palatine aponeurosis (Figure 4D)

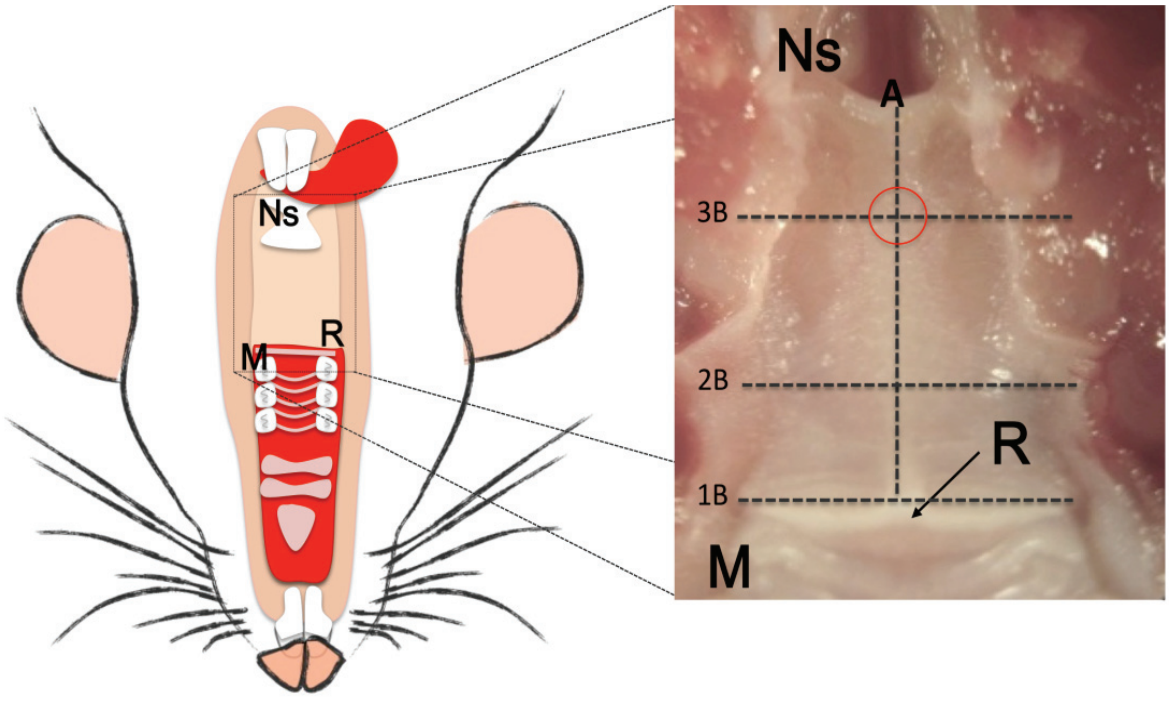

Figure 2. General aspect of the soft palate in rats. Left: Schematic representation of the soft palate (intraoral view). Right: The soft palate in the rat extends from the posterior edge of the hard palate (9th ruga) towards the nasopharyngeal sphincter. In adult rats, the length of the soft palate is about 11 mm. Ns: nasopharygeal sphincter, R: 9th ruga, M: molar. The red circle indicates the location of the excisional wound (1 mm ø). The dotted lines indicate the location of the histological sections shown in Figure 5. S: midsagittal section. B1, B2, and B 3 coronal sections.

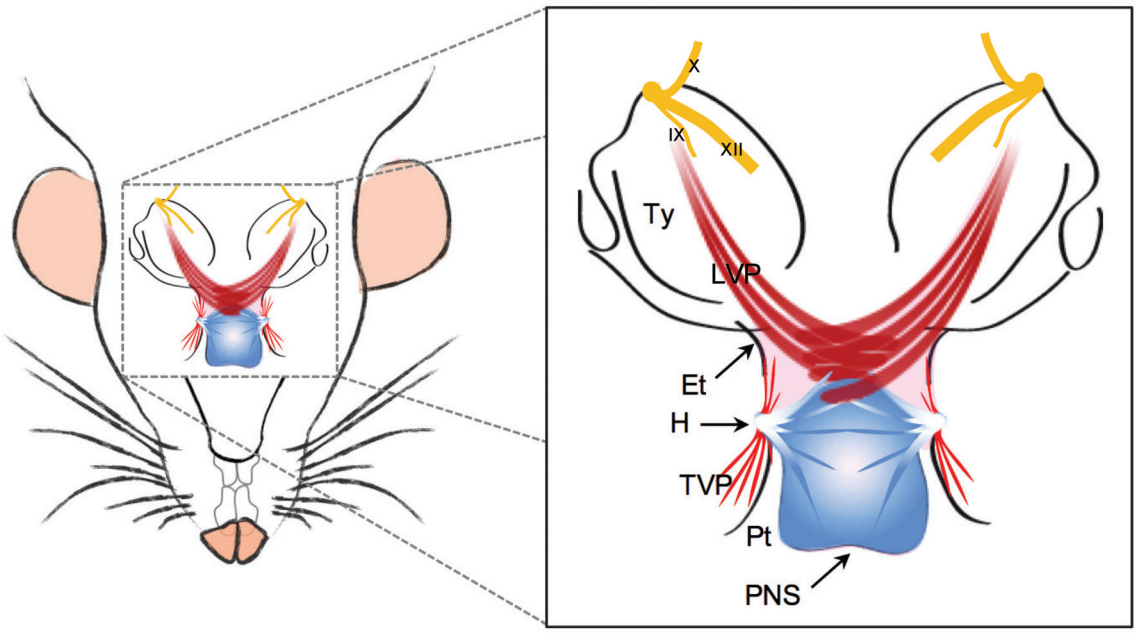

Figure 3. Schematic representation of the levator veli palatini and tensor veli palatini muscles. LVP: levator veli palatini muscle, TVP: tensor veli palatini muscle, IX: glossopharyngeus nerve, X: vagus nerve, XII: hypoglossus nerve, Ty: Tympanic bulla, At: auditory tube, Et: eustachian tube, H: pterygoid hamulus, Pt: pterygoid process, PNS: posterior nasal spine.

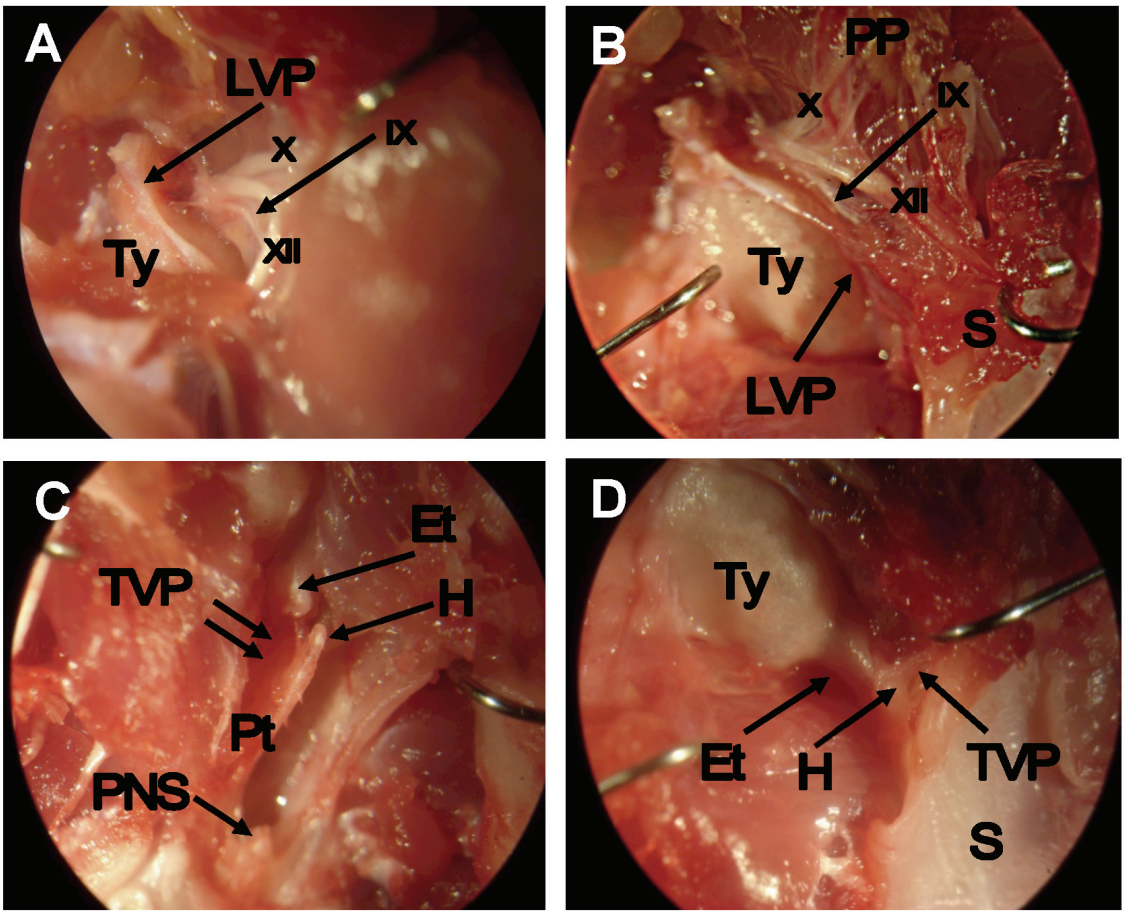

Figure 4. Anatomy of levator veli palatini and tensor veli palatini. (A) The levator veli palatini muscle arises from the inferior surface of the temporal bone, lateral and posterior from the tympanic bulla. The tympanic bulla is a bony projection of the temporal bone containing the tympanic cavity. (B) Posterior to the pterygoid process, the levator veli palatini continues towards the soft palate. The glossopharyngeal, vagus and hypoglossal nerves are visible between the levator veli palatini and the skull base. (C) The tensor veli palatini originates from the inferior surface of the sphenoid bone, the lateral surface of the perygoid plate, and the auditory tube. (D) The tendon of the tensor veli palatini turns around a curved process; the pterygoid hamulus. It continues medially towards the soft palate and forms the palatine aponeurosis. LVP: levator veli palatini muscle, TVP: tensor veli palatini muscle, IX: glossopharyngeus nerve, X: vagus nerve, XII: hypoglossus nerve, Ty: Tympanic bulla, Et: eustachian tube, H: pterygoid hamulus, Pt: pterygoid process, PNS: posterior nasal spine, S: soft palate.

Histology of the Soft Palate

Two main areas were identified in the soft palate, an anterior and a posterior area (Figure 5A). The anterior area is characterized by a thick layer of salivary glands, which is covered by a layer of oral (bottom) and nasal mucosa (top) (Figure 5B-2).

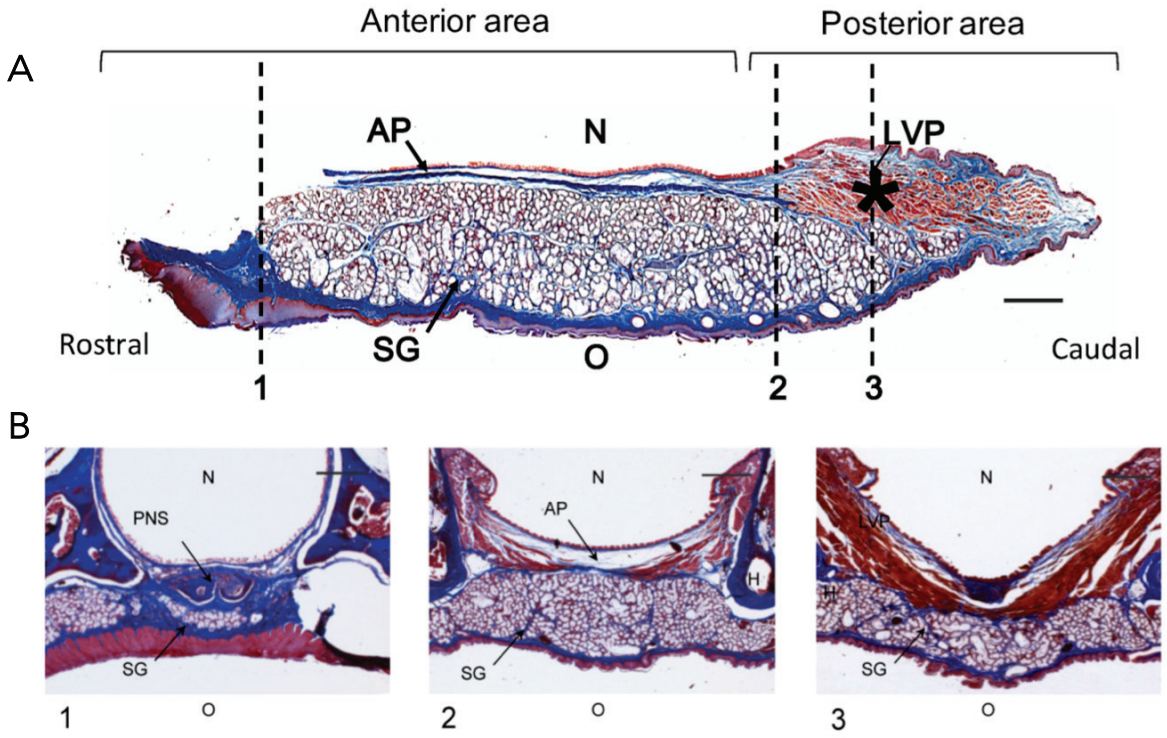

Figure 5. Histology of the soft palate. Paraffin sections were cut from the tissue and stained with AZAN. (A) Midsagittal section of the soft palate. The anterior two thirds of the soft palate mainly contain salivary glands. The posterior third of the soft palate contains an additional layer of muscle tissue. (B) Coronal sections of the soft palate. The dotted lines in figure A indicate the position of the coronal sections. (B1) The palatine aponeurosis is the continuation of the tensor veli palatini and inserts into the posterior nasal spine. (B2) Posterior to the pterygoid hamulus, the levator veli palatini fibers insert into the palatine aponeurosis. (B3) Most of muscle fibers of the levator veli palatini cross the midline and form a sling suspended from the skull base. N: nasal cavity, O: oral cavity, PA: palatine aponeurosis, SG: salivary glands, PNS: posterior nasal spine, LVP: levator veli palatini muscle, H: pterygoid hamulus. The bar represents 500 µm.

The oral surface is covered by a keratinized stratified squamous epithelium, while the nasal surface is covered by a pseudostratified ciliated columnar epithelium. The oral mucosa contains a thick collagenous submucosa. A thinner dense layer of collagenous tissue is present under the nasal epithelium; the palatine aponeurosis (Figure 5A). This is the continuation of the tensor veli palatini muscle, and inserts into the bony posterior nasal spine (Figure 5B-1). In the posterior area of the soft palate, the nasal epithelium changes into a stratified squamous epithelium (Figure 5A). In addition, a layer of muscle tissue is present.

Posterior to the bony pterygoid hamulus, the levator veli palatini muscle fibers insert into the palatine aponeurosis (Figure 5B-2). However, most of the levator veli palatini fibers cross the midline forming a muscle sling suspended from the skull base (Figure 5B-3). The nasopharyngeal sphincter is formed by the most posterior muscle fibers in the soft palate. The sphincter mainly contains fibers from the palatoglossal and palatopharyngeal muscles. The thick glandular layer gradually becomes thinner towards the posterior part of the soft palate (Figure 5A).

Wound Healing in the Soft Palate

Full-thickness defects (1 mm ø) were made in the soft palate of six rats (group 3), and left to heal for seven days. In addition, the soft palate of two other animals (group 4) was used as controls. All animals survived surgery, and showed no weight loss. After seven days, all wounds showed clinical healing. In the controls, both a layer of muscle tissue, and a layer of salivary glands are present (Figure 6A left). The wounded palates show loss of the major parts of these layers (Figure 6A right). Collagenous granulation tissue is present in the wound area, and only limited regeneration of salivary glands and muscles has occurred (Figure 6A).

ASMA is a marker for myofibroblasts. Myofibroblasts were not present in the controls tissue of the controls, except in blood vessels and salivary glands (Figure 6B). In contrast, large numbers of

myofibroblasts were present in the wound area at 7 days in the experimental group. In both groups, almost all myofibers were of the fast-twitch type (Figure 6C). More activated SCs (Pax7-, MyoD-, and MyoG-positive) were present in regenerating muscle fibers in the wound edges than in control muscle (Figure 7A, B, and C).

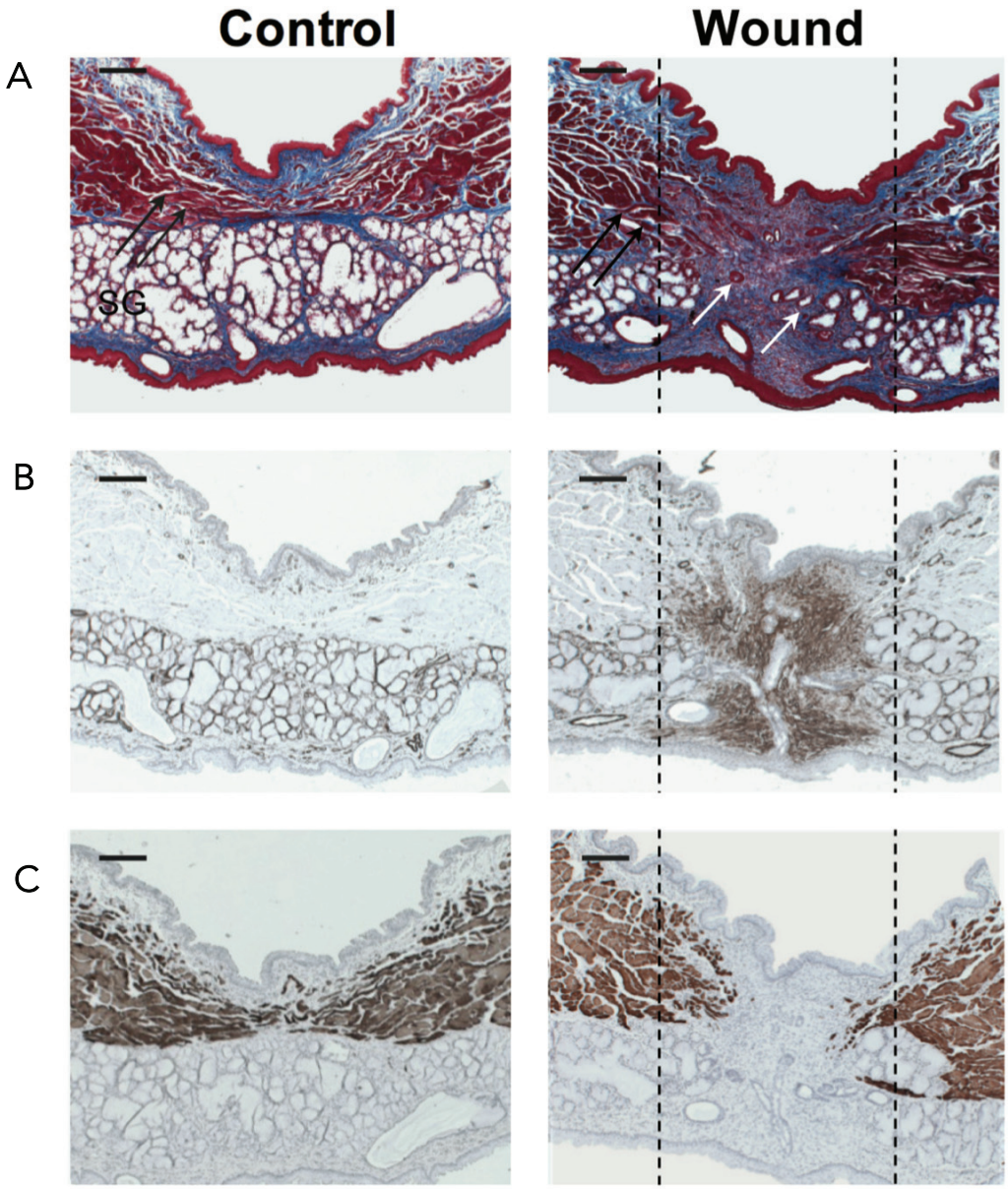

Figure 6. Regeneration of the soft palate after wounding. Control and wound tissues from the soft palate were stained with AZAN, and with antibodies against myofibroblasts (ASMA) and fast muscle fibers (Fast MyHC). After 7 days, extensive granulation tissue with collagen and myofibroblasts had formed. (A) AZAN staining. Connective tissue is stained blue, muscle tissue red. Black arrows indicate muscle fibers, white arrows indicate initial regeneration of salivary glands. SG: Salivary glands. (B) ASMA (Brown). Myofibroblasts were not present in the controls, except in blood vessels and salivary glands. In contrast, large numbers of myofibroblasts were present in the wound area at 7 days in the experimental group. (C) Fast MyHC (brown). In both groups, almost all myofibers were of the fast-twitch type. Wound margins are indicated by the dotted lines. The bar represents 200 µm.

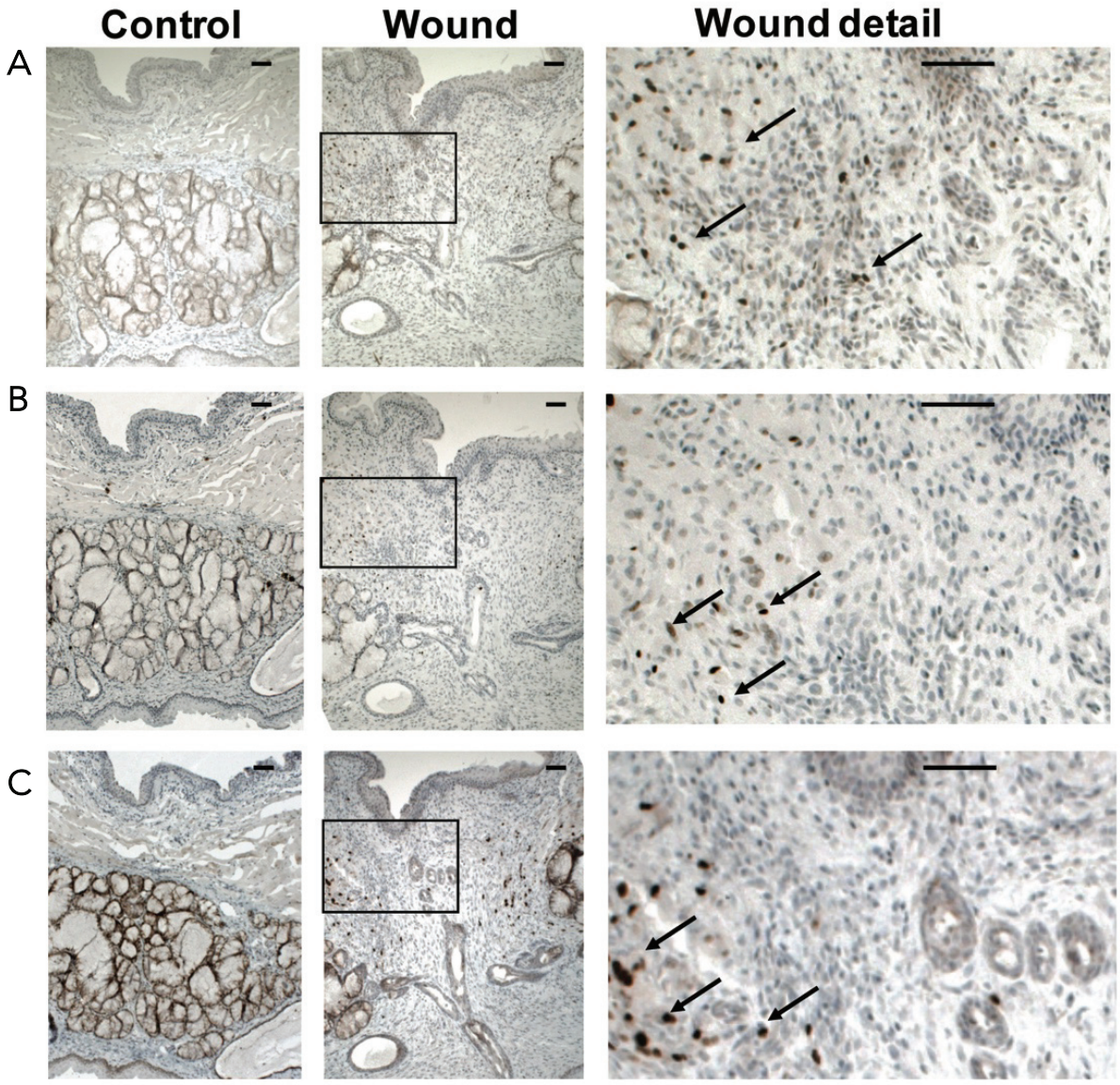

Figure 7. Satellite cells and myofibers after wounding. Control and wound tissues were stained with antibodies against Pax7, MyoD, and MyoG. Large numbers of activated satellite cells and regenerating myofibers are present in the experimental group. (A) Satellite cells (arrows) express the transcription factor Pax7. (B) The myogenic determination factor 1 (MyoD, arrows) is expressed during satellite cell proliferation. (C) Differentiation is marked by a decline in Pax7 expression, and the induction of myogenin (MyoG, arrows). Pax7-, MyoD-, and MyoG positive cells are stained brown. The bar represents 50 μm.

Discussion

Speech abnormalities are commonly observed in children with a history of a cleft in the soft palate. This can have a devastating effect on their interaction with other children and adults.[15] The regeneration of muscles in the soft palate after surgery may be hampered because of (1) their low intrinsic regenerative capacity, (2) the specific muscle properties related to clefting, and (3) the development of fibrosis.[41] The outcome of surgery may be improved by novel strategies based on tissue engineering.

A suitable animal model is required to design and optimize new strategies for soft palate repair. To our knowledge, we present the first model for the regeneration of the soft palate following surgical wounding. Our study shows that the anatomy and histology of the soft palate muscles of the rat are largely comparable to those of humans. The levator veli palatini and tensor veli palatini muscles in the rat were shown to have a similar origin and course as its homologous in humans.[43–45] In contrast, the muscular component of the soft palate in rats occupies only the posterior third of the tissue,[44] while in humans this is about half. The levator veli palatini is the most important muscle for the elevation of the soft palate in both humans and rodents.[40,42,44] Together, the tensor veli palatini and the levator veli palatini also control the passage of air through the auditory tube.[46,47] The widespread use of rodents in biomedical research ensures the availability of antibodies for specific immune staining. In combination with the ease of handling and low costs. this makes the rat the most suitable animal model.

Our model consists of a full-thickness defect in the soft palate that creates a temporal communication between the nasopharynx and the oropharynx, and thus increases the risk of bronchoaspiration. Therefore, the animals were closely monitored during the seven days after surgery. No complications were observed during this period.

Extensive collagen deposition and initial regeneration of muscle fibers and salivary glands was demonstrated after 7 days. Collagen is deposited by fibroblasts in the granulation tissue. It has been previously shown that fibroblasts proliferate in close association with satellite cells and regenerating myofibers.[48] Fibroblasts then stimulate the proliferation of satellite cells during the early phase of regeneration. Later in the regeneration process, the number of fibroblasts normally decreases to allow the formation of new muscle fibers. Growth factors such as transforming growth factor-β1 (TGF-β1) induce the differentiation of fibroblasts into myofibroblasts, which produce large amounts of extracellular matrix.[49,50] In addition, myofibroblasts induce wound contraction. The prolonged presence of myofibroblasts in the wound leads to fibrosis.[29,49] Since activated SCs do not migrate into fibrotic tissue, this may impair muscle regeneration and functional recovery of the muscle tissue after injury.[51]

Both fast- and slow-twitch fibers have been found in human soft palate muscles.[52,53] In contrast, we mainly found fast-twitch fibers in the rat soft palate. However, slow-twitch fibers were also found in the more lateral parts of the levator veli palatini muscle in rats.[54] In our study, Pax7-, MyoD-, and MyoG-positive cells were found at the wound edges, demonstrating the presence of activated SCs and differentiating myofibers. In limb muscles, activated SCs and regenerating myofibers also appear after about one week.[48,51,55,56] In limb muscles, SCs proliferate extensively within the first 2–3 days after injury.[57] At about 5 days after injury, activated SCs differentiate into myoblasts and fuse, which leads to restoration of the injured muscle within 10 days.[57] In contrast, injured head muscles do not restore within 12 days.[38] This indicates that the regeneration of head muscles is slower than that of limb muscles. Thus, further studies are necessary in order to fully characterize the regeneration of soft palate muscles.

Conclusion

The presented rat model is suitable to study muscle regeneration in the soft palate after surgical injury, and allows the development of novel adjuvant strategies to promote muscle regeneration. This offers new perspectives for the treatment of CLP patients, and for various other conditions in which the regeneration of head muscles is compromised.

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CHAPTER 4.

FIBROSIS IMPAIRS THE FORMATION OF NEW MYOFIBERS IN THE SOFT PALATE AFTER INJURY

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Abstract

Muscle repair is a crucial component of palatoplasty but little is known about muscle regeneration after cleft palate repair. We hypothesized that the formation of new myofibers is hampered by collagen accumulation after experimental injury of the soft palate of rats. One millimeter excisional defects were made in the soft palates of 32 rats. The wound area was evaluated after 3, 7, 28, and 56 days using azocarmine G and aniline blue to stain for collagen and immunohistochemistry to identify myofibroblasts and to monitor skeletal muscle differentiation. To evaluate age effects, 16 unwounded animals were evaluated at 3 and 56 days. Staining was quantified by image analysis, and one-way ANOVA was used for the statistical analysis. At day 56, the area percentage of collagen-rich tissue was higher in the injured palatal muscles (46.7±6.9%) than in non-wounded controls (15.9±1.0%, $p<0.05$). Myofibroblasts were present in the injured muscles at days 3 and 7 only. The numbers of proliferating and differentiating myoblasts within the wound area were greater at day 7 ($p<0.05$), but only a few new myofibers had formed by 56 days. No age effects were found. The results indicate that surgical wounding of the soft palate results in muscle fibrosis. Although activated satellite cells migrated into the wound area, no new myofibers formed. Thus, regeneration and function of the soft palate muscles after injury may be improved by regenerative medicine approaches.

Velopharyngeal dysfunction is common after surgical repair of the soft palate.[1] Several causative factors have been proposed, including age at surgery, the skills and experience of the surgeon, cleft type and extension, repair technique, and fibrosis.[2-5] Muscle fibrosis seems to be a major factor. Although muscle repair is an important component of contemporary palatoplasty,[4] little is known about muscle regeneration after cleft palate repair.

The ability of skeletal muscle to regenerate through the action of satellite cells (SCs) is well established.[6] Upon injury, SCs become activated and migrate to the site of injury, where they proliferate, differentiate into myoblasts, and fuse to form new myofibers or repair damaged ones.[7] Quiescent SCs express the transcription factor Pax7;[8] proliferating myoblasts also express the myogenic determination factor 1 (MyoD).[9] Differentiating myoblasts start to express myogenin (MyoG)[10] and terminal differentiation is marked by the expression of muscle-specific proteins, such as fast myosin heavy chain (MyHC).[11]

Several experimental strategies have been used in regenerative medicine to improve muscle regeneration.[12] Most of these studies have been performed on limb or trunk muscles, and studies on branchiomeric muscles are scarce. Branchiomeric muscles originate from the branchial arches and include the masseter and levator veli palatini muscles. Branchiomeric muscles have been suggested to contain fewer SCs and regenerate slower than limb muscles.[13] Compared to limb muscles, more fibrous connective tissue seems to form when branchiomeric muscles heal.[14] Proliferating SCs from branchiomeric muscles have a different transcription factor profile than limb muscle.[13] Moreover, muscles in the soft palate of cleft patients are usually smaller and less well-organized than palatal muscles from healthy subjects.[15,16] All of these factors may

contribute to the poor regeneration of the soft palate muscles after surgical cleft closure.[17]

Therefore, it is crucial to characterize the regeneration of the soft palate muscles after injury and to develop specific strategies for improving muscle regeneration. In this study, we investigate regeneration of the soft palate muscles and the formation of fibrotic tissue after excisional palatal injury in a rat model.

Material and Methods

Animals

The protocol was approved by the local Board for Animal Experiments at Radboud University Nijmegen in accordance with Dutch laws and regulations (RU-DEC-2011-125). Forty-eight adult male Sprague Dawley rats weighing 280–300 g (Harlan BV, Horst, the Netherlands) were housed under standard laboratory conditions. The rats had been acclimatized to the animal facility for one week before the start of the experiments. The animals were randomized into six groups (n=8, Table 1). Groups 1 and 6 were used as unwounded age controls, whereas groups 2-5 were used for surgical wounding (Table 1).

Table 1. Groups and time points

Group	Type	Time point	*n*
1	Age control	3 days	8
2	Experimental	3 days	8
3	Experimental	7 days	8
4	Experimental	28 days	8
5	Experimental	56 days	8
6	Age control	56 days	8

Excisional wounding of the soft palate

As previously described,[18] the animals received buprenorphine (0.02 mg/kg s.c.; Temgesic, Schering Plough, Brussels, Belgium) as an analgesic before surgery and at 12-h intervals for the next 2 days. Briefly, animals in groups 2-5 were anesthetized and placed on an operating table in a supine position for surgery. An excisional wound (1 mm ø) was made 7 mm behind the ninth palatal ruga using a biopsy punch. After reversion of the anesthesia and over the following 3 days the animals received powdered chow in water. Their behavior was monitored daily with special attention to water and food intake, weight loss, and activity. Animals were euthanized using the standard CO_2/O_2 protocol after 3, 7, 28, and 56 days.

Histology

The soft palate of each animal was dissected, fixed in 4% paraformaldehyde (PFA) in PBS, and processed for paraffin embedding. Paraffin sections were stained with azocarmine G and aniline blue (AZAN) to identify connective tissue/collagen (blue) and muscle tissue (red).

Immunohistochemistry

Sections were stained as described previously.[18] The following primary antibodies were used: mouse anti-Pax7 (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), mouse anti-MyoD (1:50; DAKO, Dakopatts, Glostrup, Denmark), mouse anti-myogenin (1:100; DSHB), mouse anti-alpha-smooth muscle actin (α-SMA, 1: 10,000; Chemical Co., St Louis, MO, USA), and mouse anti-fast MyHC (1:5000; Sigma Chemical Co.).

Quantification

All AZAN- and antibody-stained sections were visualized using a Zeiss microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) and photographed. The muscle layer was digitally isolated from

each photograph using Adobe Photoshop (CS5 Extended 12.0x32; Adobe Systems, San Jose, CA). Subsequently, two areas of interest were demarcated, a central area corresponding to the wound area (700 µm) and two lateral areas on both sides of the wound (500 µm per side, Figure 1). For AZAN, α-SMA, and MyHC staining, the percentage of stained area was determined using a macro in Fiji.[19] The number of Pax7-, MyoD-, and MyoG-positive cells was counted in both the central and lateral areas and expressed as cells/mm^2. All data are expressed as the mean percentage ± SD of the total area.

Data analysis and statistics

All data were analyzed by one-way ANOVA and a Kruskal-Wallis post-hoc test. $p<0.05$ was considered significant. Differences between the experimental and age control groups were analyzed at days 3 and 56. Differences between all experimental groups were analyzed on days 3, 7, 28, and 56. Differences between the two age control groups at were analyzed at days 3 and 56.

Results

Clinical and histological observations following palatal injury

Pilot studies in rats revealed that incisional wounding of the soft palate results in full muscle regeneration, whereas excisional wounding (full-thickness defects of 1 mm Ø) impairs healing. We chose the latter wound model because the outcome of excisional wounding is more comparable to clinical cleft palate repair.

One animal (group 2) died during the surgical procedure due to oronasal obstruction. On day 7, all wounds demonstrated clinical healing. However, after histological analysis we found that six wounds had not closed completely, but had developed an oronasal communication lined with epithelium. These excisional wounds may have been made too close to the nasopharyngeal sphincter, and its

contractile activity may have counteracted wound closure. These specimens from groups 4 (*n*=3) and 5 (*n*=3) were excluded from further analysis. Two main layers are present in the soft palate of the rat: an upper layer containing the muscles and a lower layer containing the salivary glands (Figure 1). The oral surface is covered by a keratinized stratified squamous epithelium, whereas the nasal surface is covered by a pseudostratified ciliated columnar epithelium. After digital isolation of the muscle layer, three areas of interest were chosen: one central area and two lateral areas. All measurements were performed in these areas (Figure 1).

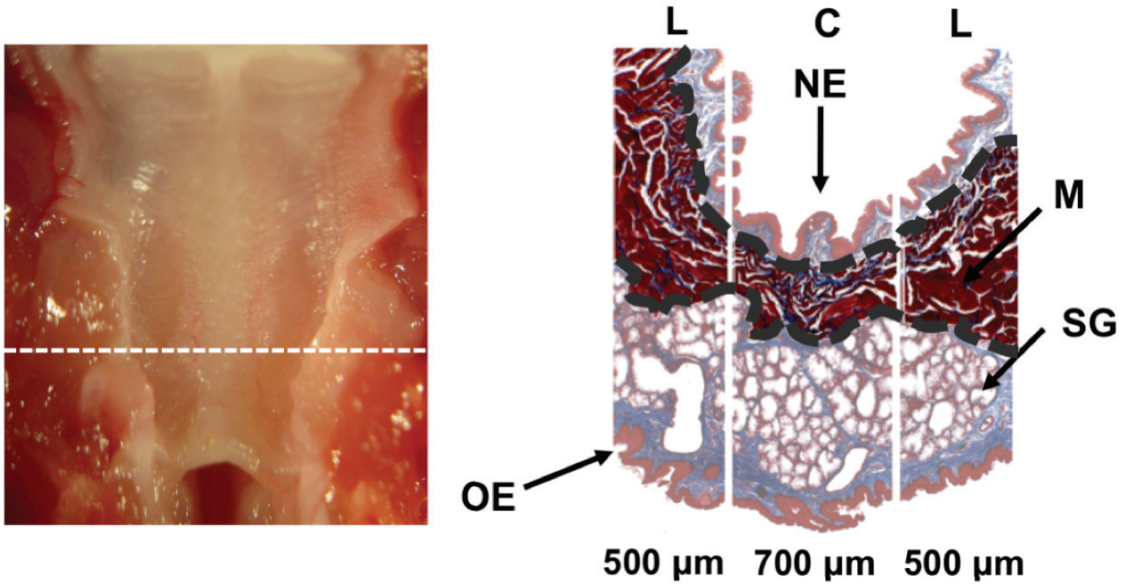

Figure 1. Regions of interest. This figure shows both the dissected soft palate (left) and a frontal AZANstained section (right) for the control group on day 3. The dotted white line indicates the level of the histological section (left). Two main layers are present in the soft palate: an upper layer containing the muscles (area between black dotted lines) and a lower layer containing the salivary glands. All AZAN- and antibody-stained sections were photographed, and the muscle layer was isolated digitally. Next, two areas of interest were identified, a central area corresponding to the wound area (700 µm) and two lateral areas on both sides of the wound (500 µm per side). PNS: tip of posterior nasal spine, Ns: nasopharyngeal sphincter, L: lateral area, C: central area, NE: nasal epithelium, M: muscle, SG: salivary glands. and OE: oral epithelium.

Collagen quantification and myofibroblast staining

To measure collagen deposition, sections were stained with AZAN (Figure 2). After full thickness wounding, the muscle and salivary gland layers were completely lost. By day 3 the wound was filled with a blood clot, which was replaced with granulation tissue by day 7 (not shown). By day 56 the wound area was occupied by collagenous tissue (Figure 2, *left and center*). Over time, the amount of collagen (Figure 2, *right*) in the central area increased from 18 ± 4.0% on day 7 to 46.7 ± 6.9% on day 56 ($p<0.05$). The amount of collagen in the central area was significantly greater in the experimental group (46.7 ± 6.9%) than in the age control (15.9 ± 1.0%, $p<0.05$) at day 56. No significant differences were found between the age controls at days 3 and 56.

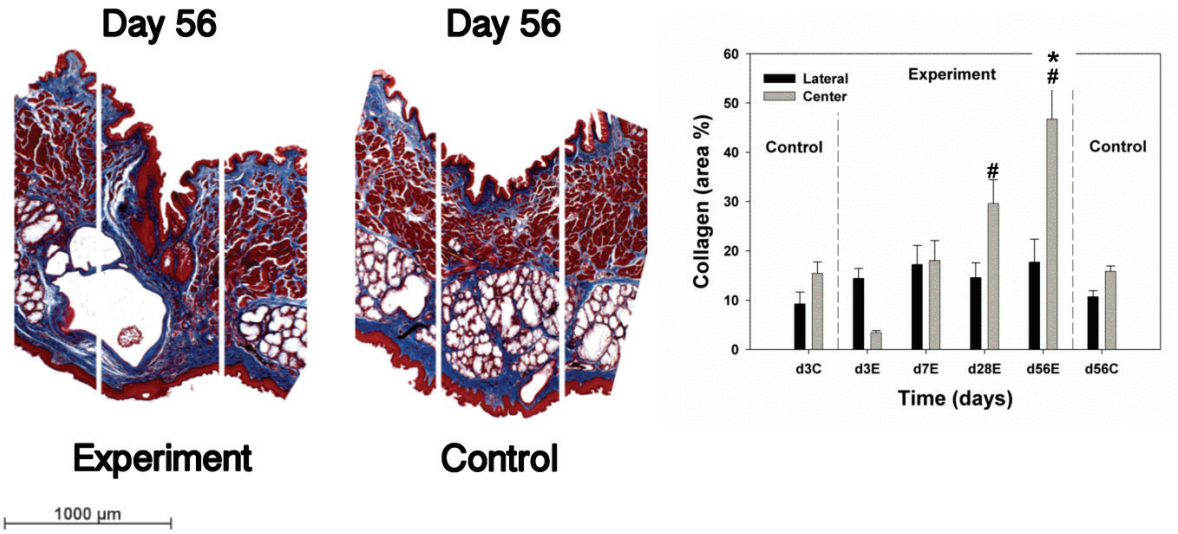

Figure 2. AZAN staining and quantification. Control and wound sections from the soft palate were stained with AZAN to discriminate collagen from muscle tissue (area between white dotted lines). Sections from experimental (left) and control (center) animals on day 56. Quantification of the amount of collagen (right) is expressed as the mean percentage ± SD of the total area. #$p<0.05$ between the experimental groups, *$p<0.05$ between experimental and control groups at day 56.

To identify myofibroblasts, paraffin sections were stained with antibodies against α-SMA (Figure 3). In the age control groups, α-SMA-positive cells were only present in blood vessels and the myoepithelium of the salivary glands. In the experimental groups, myofibroblasts were present in the lamina propia of the nasal and oral mucosa already by day 3, but also in the muscular and glandular layers by day 7 (Figure 3, *left*). By days 28 and 56, only blood vessels and myoepithelial cells were present (Figure 3, *center*). At day 3, the α-SMA-positive area (Figure 3, *right*) in the experimental group was significantly larger in both the central and lateral areas (lateral: 14.4 ± 7.0%, center: 25.5 ± 9.4%, $p<0.05$) compared to the controls (lateral: 4.2 ± 1.3%, center: 5.5 ± 0.9%, $p<0.05$). Myofibroblasts were present in the central area on day 7 and significantly decreased from 32.0 ± 8.5% to 7.3 ± 1.5% by day 56 ($p<0.05$). No significant differences were found between the age controls at days 3 and 56.

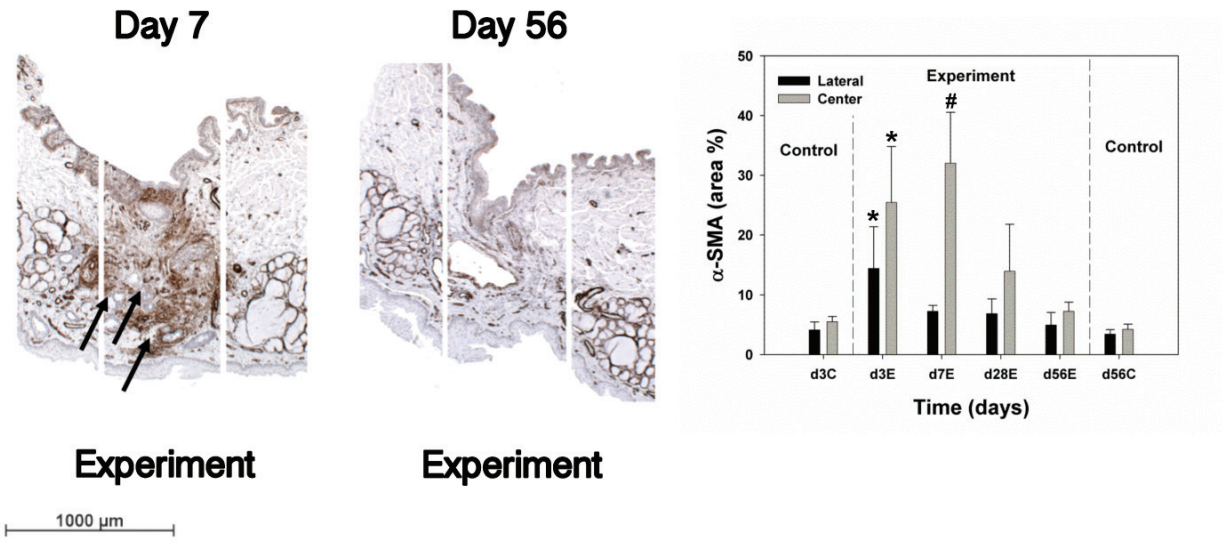

Figure 3. α-SMA immunostaining and quantification. Control and wound sections were stained with antibodies against α-SMA to identify myofibroblasts (arrows). Sections from wounds on day 7 (left) and day 56 (center) are shown. The quantification of α-SMA (right) is expressed as the mean percentage ± SD of the total area. #p<0.05 between the experimental groups, *p<0.05 between experimental and control groups at day 3.

Satellite cell activation

Sections were stained with antibodies against Pax7 to identify SCs. SCs first appeared in the lateral area by day 3 and in the central area by day 7 (Figure 4, *above, left*). The number of SCs increased from day 3 to day 7 and then decreased up to day 56. The number of SCs is illustrated in Figure 4 (*above, right*). Only a few SCs were present in the controls. The number of SCs was 2-fold higher in the lateral area of the experimental group than in the age control group at day 3 ($p<0.05$). In addition, the number of SCs in the experimental group was significantly higher in both areas at day 7 (central: 1.6-fold, lateral: 4.7-fold) compared to day 3 ($p<0.05$). No significant differences were found between the age controls at 3 and 56 days.

Satellite cell proliferation

Sections were stained with antibodies against MyoD to identify proliferating SCs. Proliferating SCs first appeared in the lateral area by day 3 and in the central area by day 7 (Figure 4, *center, left*). The number of proliferating SCs increased from day 3 to day 7 and then decreased to day 56. The number of proliferating SCs is illustrated in Figure 4 (*center, right*). Only a small number of proliferating SCs were present in the controls. The number of proliferating SCs was 10-fold higher ($p<0.05$) in the lateral area of the experimental group than in the age control group at day 3. In addition, on day 7 the number of proliferating SCs was 4.3- and 6.8-fold higher ($p<0.05$) in the central and lateral areas, respectively, compared to the experimental group on day 3. No significant differences were found between the age controls at 3 and 56 days.

Satellite cell differentiation

Sections were stained with antibodies against MyoG to evaluate the differentiation of SCs. As expected, differentiating SCs first appeared in the lateral area by day 3 and the central area by day 7

(Figure 4, *below, left*). The number of differentiating SCs increased from day 3 to day 7 and then decreased to day 56. The number of differentiating SCs is illustrated in Figure 4 (*below, right*). Only a few differentiating SCs were present in the controls. The number of differentiating SCs was 143-fold higher ($p<0.05$) in the lateral area of the experimental group compared to its age control group at day 3. On day 7 the number of differentiating SCs was 1.4- and 3.1-fold higher ($p<0.05$) in the central and lateral areas, respectively, compared to the experimental group at day 3. No significant differences were found between the age controls at 3 and 56 days.

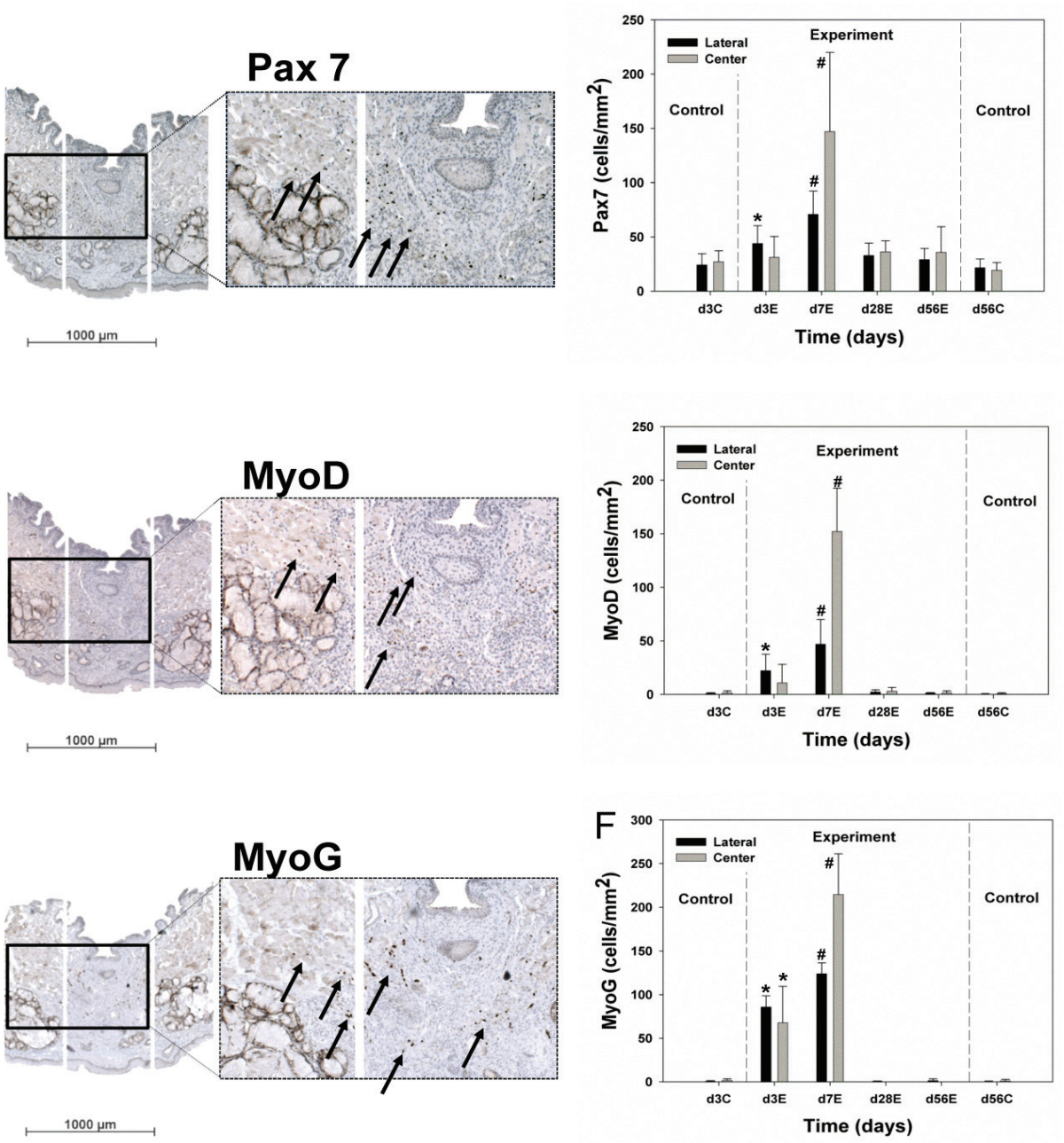

Figure 4. Pax7, MyoD, and MyoG immunostaining and quantification. Sections were stained with antibodies against Pax7 (above, left), MyoD (center, left), and MyoG (below, left) on day 7 to evaluate activated SCs, proliferating SCs, and differentiating myoblasts, respectively. The number of cells positive for Pax7 (above, right), MyoD (center, right), and MyoG (below, right) was quantified and expressed as cells/mm2 ± SD of the total. #p<0.05 between the experimental groups, *p<0.05 between experimental and control groups at day 3.

Myofiber formation

To identify myofibers, sections were stained with antibodies against fast MyHC (Figure 5). At 56 days, fewer myofibers were present in the central area of experimental animals compared to the controls (Figure 5, *left and center*). At day 3, the MyHC-positive area (Figure 5, *right*) in the experimental group was significantly smaller in both the lateral and central areas (lateral: 47.1 ± 8.9%, central: 1.4 ± 2.8%) compared to the age control (lateral: 66.8 ± 4.6%, central: 67.1 ± 3.7%, $p<0.05$). In the experimental groups, the MyHC-positive area in the center increased from 12.8 ± 6.8% on day 6 to 28.2 ± 5.9% on day 56 ($p<0.05$). However, by day 56, the MyHC area was significantly smaller (28.2 ± 5.9%) in the experimental groups than in the age controls (56.5 ± 4.4%, $p<0.05$). No significant differences were found between the age controls at 3 and 56 days.

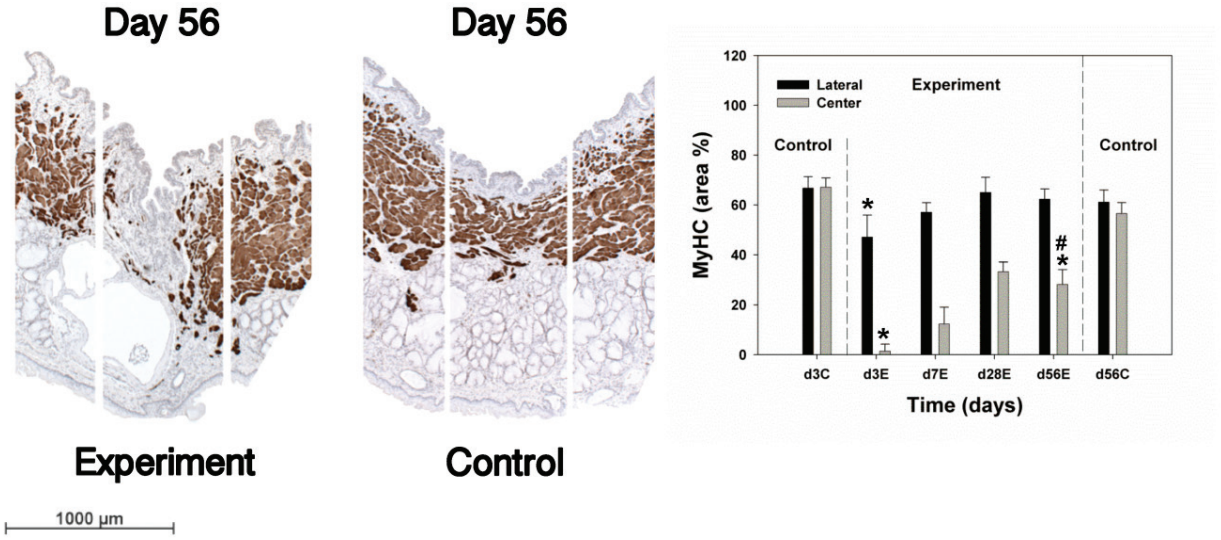

Figure 5. MyHC staining and quantification. Sections were stained with antibodies against fast MyHC to identify myofibers. Sections from experimental (left), and control (center) animals at day 56 are shown. Quantification of the amount of MyHC (right, area between black dotted lines) is expressed as the mean percentage ± SD of the total area. #p<0.05 between the experimental groups, *p<0.05 between experimental and control groups at day 56.

Discussion

The aim of this study was to evaluate the regeneration of myofibers and the development of fibrotic tissue after injury. We previously demonstrated that the anatomy and histology of the soft palate muscles of the rat are largely comparable to that of humans.[18] Here, we show for the first time that muscle regeneration in the soft palate of the rat after excisional injury is incomplete. Extensive fibrotic tissue developed with little or no formation of new myofibers. Therefore, the original architecture of the muscle layer was not restored. First, the wounds filled with a blood clot, which was gradually replaced by granulation tissue that developed into fibrotic tissue.

In our study, activated SCs and proliferating and differentiating SCs appeared early during wound healing, but myotube formation did not occur in the first week after injury. Similar results were found in a full-thickness wound model in the limb muscles of rats.[20] However, in that study no SCs were found within the wound area at any time point, which may be related to the muscle type or larger defect size (2 mm vs. 1 mm Ø). In other types of limb muscle injury without a tissue defect, such as freezing, crushing, or the application of myotoxic drugs, the muscle damage is not as extensive as in our model. In these models, the SCs proliferate extensively during the first few days and new myotubes appear within one week.[21] Previous studies suggest that SC proliferation in branchiomeric muscles takes longer than in limb muscles, preventing adequate muscle regeneration.[13,14] Therefore, care must be taken when comparing studies in different muscles.

We also observed that only a few new myofibers had formed after 56 days despite the early presence of differentiated SCs. In addition, collagen-rich tissue had developed in the wound area. Transforming growth factor-β1 (TGF-β1) and myostatin, both members of the TGF-β super family of growth factors, can induce

fibrosis in skeletal muscle after injury. TGF-β1 plays a significant role in the initiation of fibrosis and induces the differentiation of fibroblasts into myofibroblasts, which are mainly responsible for the excessive production of collagen and other components of the extracellular matrix.[20,22] Myostatin stimulates fibroblast proliferation, reduces the migration of macrophages and myoblasts, and inhibits myoblast differentiation.[23,24] Myostatin knockout mice have significantly less fibrosis in injured muscle.[24,25] Moreover, myostatin up-regulates the expression of TGF-β1 in injured muscle, and vice versa.[25] These factors may contribute to fibrosis in our model. In our study, myofibroblasts were present early after wounding, suggesting that they are not directly responsible for fibrosis. However, because the area of damaged tissue is large and revascularization may be compromised, the ischemic conditions may favor fibroblast proliferation and scar tissue formation.[26] In our study and others, SCs seem to actively migrate into the injured area[27,28]; however little or no formation of new myofibers in the wound area were evident after 56 days. Regenerating muscle fibers generally develop along the basal lamina of the injured muscle.[29,30] In a full-thickness defect like our model, the cell membrane and basal lamina of the muscle fibers are completely lost. Without the guidance of these structures, the regeneration of muscle fibers is probably impaired further.[30-32] In addition, disturbed myoblast fusion could result from impaired cell-extracellular matrix (ECM) signaling because of changes in the ECM.[33,34] However, further studies are warranted to better understand how the ECM, the cell membrane and the basal lamina affect SC activity and fusion.[29,35] Thus, ischemia, collagen accumulation, the loss of structural guidance, and changes in the ECM may hamper myoblast fusion and myofiber formation.

In the clinic, the three major factors that limit muscle regeneration and promote fibrosis following cleft surgery seem to include: 1) inadequate reconstruction of the levator veli palatini muscle sling,

as adequate alignment of muscles fibers ensures proper guidance for the regenerating myofibers; 2) induced trauma, limited revascularization, and re-innervation of the cleft palate muscles, as the duration of surgery and extensive mobilization of the dissected muscles during soft palate repair compromise vascularization, which is essential for the early events in regeneration, such as the inflammatory response[36]; and 3) intrinsic muscle abnormalities associated with the cleft, such as atrophy and reduced capillary supply, which may contribute to suboptimal regeneration. Excessive lateral dissection of the levator veli palatini can damage the innervation of the soft palate muscles.[37] Innervation is initially not required for regeneration, but when the new myofibers are not nerve-activated they will fail to mature and become atrophic. Eventually, these myofibers degenerate and are replaced by connective tissue.[38,39] It is important to realize that fibrosis can increase in cases that require multiple surgical interventions.[40]

In summary, we developed a new model for fibrosis-compromised muscle regeneration in the soft palate by introducing a full-thickness defect. Muscle regeneration begins with the activation, recruitment, and proliferation of SCs from the wound margins early in wound healing. However, differentiated myoblasts within the wound fail to form new myofibers as large amounts of collagen are deposited in the wounded area. Two possible issues need to be further investigated: the loss of structural guidance by muscle membranes and the effect of fibrosis on the formation of myofibers. Proper alignment of regenerating myotubes may be achieved with scaffolds of specific design and surface topography, whereas the use of growth factors and/or ECM components may limit the development of fibrosis. This model provides a tool for developing novel strategies based on tissue engineering to improve the outcomes of cleft palate surgery. The use of isolated SCs, growth factors, and suitable scaffolds may improve muscle regeneration and function after cleft palate repair.

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"... we feel sometimes like kids playing with these molecules and seeing what are the possibilities to build."

Bernand Feringa. Nobel Prize in Chemistry, 2016

In vitro studies

Chapter 5

Isolation and Characterization of Satellite Cells from Rat Head Branchiomeric Muscles

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Abstract

Fibrosis and defective muscle regeneration can hamper the functional recovery of the soft palate muscles after cleft palate repair. This causes persistent problems in speech, swallowing, and sucking. *In vitro* culture systems that allow the study of satellite cells (myogenic stem cells) from head muscles are crucial to develop new therapies based on tissue engineering to promote muscle regeneration after surgery. These systems will offer new perspectives for the treatment of cleft palate patients. A protocol for the isolation, culture and differentiation of satellite cells from head muscles is presented. The isolation is based on enzymatic digestion and trituration to release the satellite cells. In addition, this protocol comprises an innovative method using extracellular matrix gel coatings of millimeter size, which requires only low numbers of satellite cells for differentiation assays.

About 1:500 to 1:1,000 newborns exhibit a cleft involving the lip and/or palate (CLP); thus this is the most common congenital malformation in humans.[1] The muscles of the soft palate are critical for the functioning of the soft palate during speech, swallowing, and sucking. If a cleft of the soft palate is present, these muscles are abnormally inserted into the posterior end of the palatal bone.

The soft palate moves backwards to the posterior pharyngeal wall (by retraction of the levator veli palatini muscle) during speech, preventing air to escape through the nose. Children with a cleft in the palate do not have this control function resulting in a phenomenon known as velopharyngeal dysfunction.[2,3] Although the treatment protocols are variable, surgical repair of the soft palate takes place in early childhood (6-36 months of age).[4] The abnormally inserted muscles of the soft palate can be surgically corrected,[5-7] however, velopharyngeal dysfunction persists in 7% to 30% of the patients.[2,3,8-10]

The ability of skeletal muscle to regenerate through the action of satellite cells (SCs) is well established.[11,12] Upon muscle injury, SCs are activated and migrate to the site of injury. They then proliferate, differentiate, and fuse to form new myofibers or repair damaged ones[13]. Quiescent SCs express the transcription factor Pax7,[14,15] while their progeny, the proliferating myoblasts, additionally express the myogenic determination factor 1 (MyoD).[16] Differentiating myoblasts start to express myogenin (MyoG).[17] The terminal differentiation of myoblasts is marked by the formation of myofibers, and the expression of muscle-specific proteins such as myosin heavy chain (MyHC).[16,18]

Recently, several strategies have been used in regenerative medicine to improve muscle regeneration of limb muscles.[19-23] Specific studies on branchiomeric head muscles are also important because it was recently demonstrated that they differ from other

muscles in several aspects.[24] In contrast with limb muscles, it has been suggested that branchiomeric head muscles contain less SCs,[25] regenerate slower, and more fibrous connective tissue is formed after injury.[26] In addition, proliferating SCs from branchiomeric head muscles also express other transcription factors. For instance, Tcf21, a transcription factor for craniofacial muscle formation is strongly expressed in regenerating head muscles but hardly in regenerating limb muscles.[25] The muscles in the soft palate of CLP patients are usually smaller and less well-organized compared to normal palatal muscles.[27,28] Slow and fast fibers are both present in the soft palate muscles but the slow fibers are more abundant. In contrast, cleft muscles contain a higher proportion of fast fibers and also a reduced capillary supply compared with normal soft palate muscles.[29-31] Fast fibers are more prone to contraction-induced injury.[31-33] The accompanying poor capillary supply may also promote fibrosis.[34,35] All these aspects may contribute to the poor regeneration of soft palate muscles after surgical cleft closure.[36] In view of this, a protocol for the isolation and characterization of branchiomeric head muscle SCs is crucial. This provides the possibility to study SC biology of branchiomeric head muscles.

In addition, new therapies based on tissue engineering can be developed to promote muscle regeneration after surgery in CLP and other conditions compromising the craniofacial area.

In general, SCs can be obtained after dissociation of muscle tissue.[14] Mincing, enzymatic digestion, and trituration are generally required to release SCs from their niche. SCs can be purified by pre-plating on uncoated dishes,[14,37,38] fractionation on Percoll,[39,40] or fluorescent- or magnetic cell sorting.[41-43] Here we present a new economic and rapid protocol for the isolation of satellite cells from branchiomeric head muscles of young adult rats. This protocol is based on a previous manuscript[14] and specifically adapted for small tissue samples. The isolation of SCs

from representative muscles originating from the 1st, 2nd, and 4th branchial arches are described. After isolation, low numbers of satellite cells are cultured on extracellular matrix gel spots of millimeter size to study their differentiation. This approach avoids the requirement for the expansion and passaging of SCs.

Protocol

All experiments described herein were approved by the local Board for Animal Experiments from the Radboud University Nijmegen in accordance with Dutch laws and regulations (RU-DEC 2013-205).

Extracellular Matrix Gel Spots

Perform the following steps one day before the isolation:

1. Thaw an aliquot extracellular matrix gel (100 µl) at 4°C for at least 1.5 hr. Dilute 1:10 in Dulbecco's modified Eagle's medium; with 4,500 mg/L glucose, 4 mM L-glutamine, and 110 mg/ml sodium pyruvate (DMEM). Keep the extracellular matrix gel at 4°C at all times.

Note: Abrupt temperature changes will result in uneven coating and crystal formation.

2. Keep the diluted extracellular matrix gel solution on ice for 15 min.
3. Pre-chill a 20 µl micropipette for 10 min.
4. Put 8-well chamber slides into a 100 mm Petri dish and transfer the dish onto a cold surface (e.g. a freezer pack) for 10 min.
5. Use the pre-chilled micropipette to put a drop of 10 µl extracellular matrix gel in each well. Keep the Petri dish on the cold surface for at least another 7 min (Figure 1A).

6. Completely remove the remaining extracellular matrix gel (Figure 1B), and dry the wells at 37°C overnight.

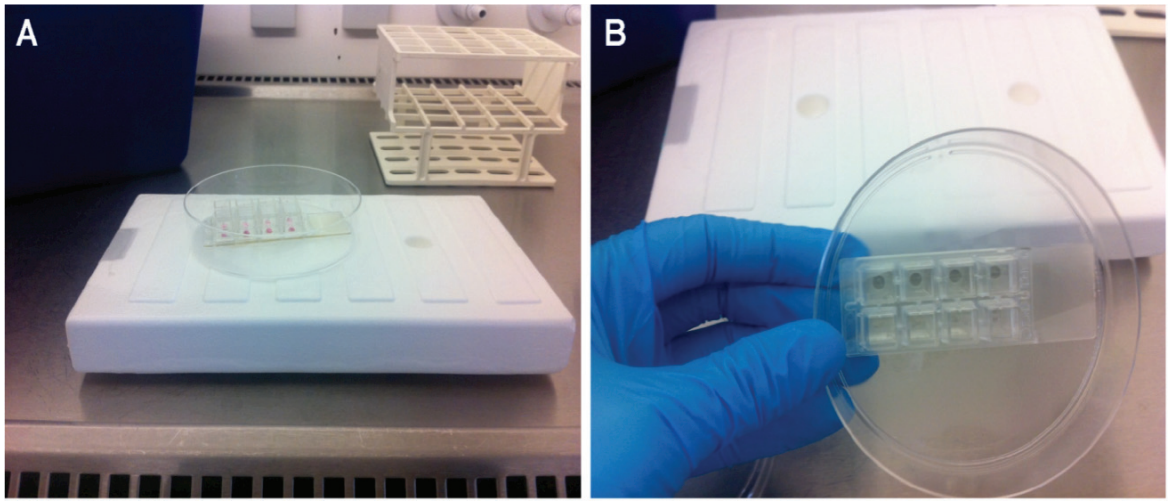


Figure 1. Extracellular matrix gel spots in a chamber slide. (A) For easy manipulation, place the 8-well chamber slide into a 100 mm Petri dish. Pipet 10 µl extracellular matrix gel in each chamber and put it on a cold surface (7 min). (B) Chamber slide after the excess extracellular matrix gel is removed.

Dissection of Head Muscles (Masseter, Digastric, and Levator Veli Palatini)

1. Before dissection, prepare 50 ml of phosphate-buffered saline (PBS) supplemented with 2% Penicillin-Streptomycin (P/S). Keep on ice.
2. After euthanasia of one young adult rat (9 weeks) with CO2/O2, decapitate the head and remove the skin from the head. Transfer the head to ice-cold PBS supplemented with 2% P/S in a 50 ml tube.
3. Masseter muscle (derived from the 1st branchial arch)

   - Place the head with one side up on a silicone pad and fix with hypodermic needles (Figure 2A).

   - Identify the parotid gland and the facial nerve (Figure 2A). Expose the deep fascia covering the gland. Cut the fascia and remove the gland using dissection scissors. Identify the external auditory canal. Trace the facial nerve from the

stylomastoid foramen and carefully remove the temporal, zygomatic, and buccal branches with a scalpel blade No. 15.

- Free the superficial head of the masseter muscle by removing the fascia. Identify both superficial and deep heads of the masseter muscle. Trace the superficial head until its thick tendinous aponeurosis inserted in the zygomatic process of the maxilla.
- Separate the tendon from its origin at the zygomatic process with a straight forceps. Cut it with a scalpel blade No. 15 or dissection scissors and carefully life it (Figure 2B).
- Dissect the superficial head of the masseter until its insertion at the angle and inferior half of the lateral surface of the ramus of the mandible with a scalpel blade No. 15 (Figure 2C). Now, completely remove the muscle.

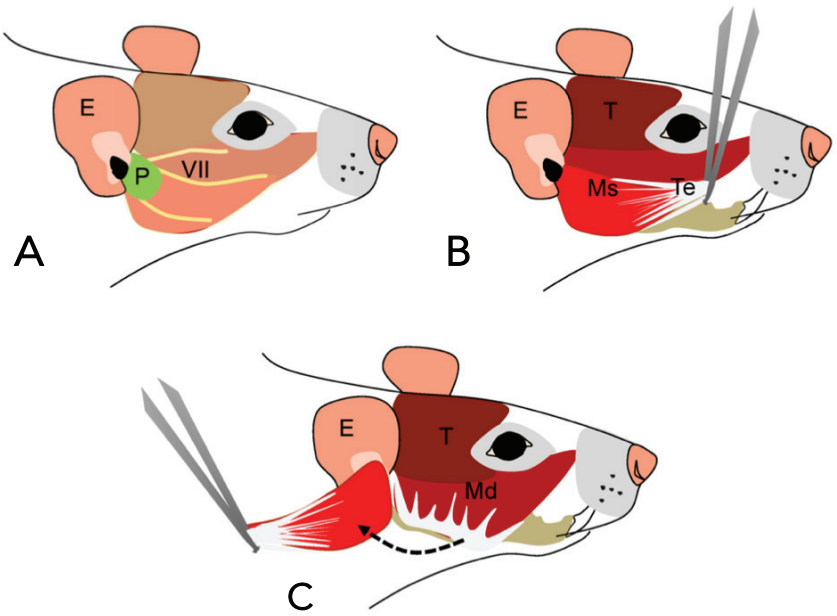


Figure 2. Dissection of the masseter muscle. (A) Head of the animal in a lateral view. Ear (E), Parotid gland (P) and facial nerve (VII). (B) Tendinous aponeurosis (Te) of the superficial head of the masseter muscle (Ms) and temporal muscle (T). Separate the tendon from its insertion with a forceps. (C) Carefully dissect the muscle until its insertion at the ramus of the mandible. E: ear, P: parotid gland, VII: facial nerve, T: Temporal muscle, Ms: superficial head of the masseter muscle, Te: tendon, Mp: deep head of the masseter muscle.

4. Posterior belly of the digastric muscle (derived from the 2nd branchial arch)

- Place the head in a supine position on the silicone pad and fix with hypodermic needles (Figure 3A).
- Remove the subcutaneous fat overlying both sublingual and submandibular glands. Next, remove the superficial fascia and glands using dissection scissors. Expose the digastric muscle (anterior and posterior belly).
- Hold the anterior tendon of the posterior belly with a straight forceps, cut it, and dissect it carefully until its origin in the tympanic bulla (Figure 3B). Do the same at the contralateral side.

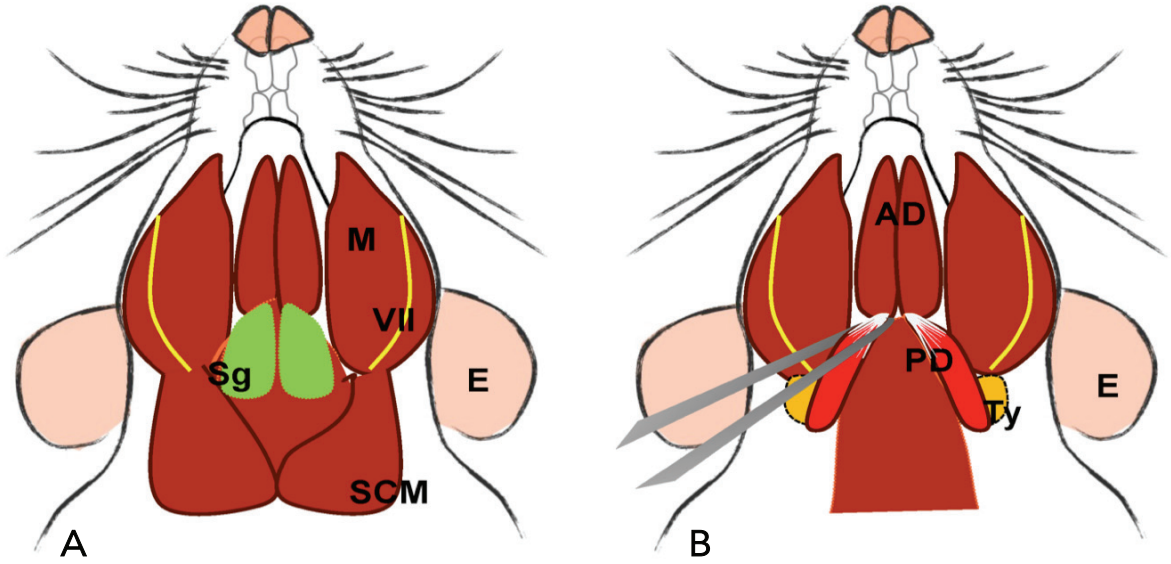


Figure 3. Dissection of the posterior belly of the digastric muscle. (A) Head of the animal in a supine position. Localize the submandibular gland (Sg), masseter muscle (M), facial nerve (VII) and sternocleidomastoid muscle (SCM). Remove the submandibular gland. (B) Localize the digastric muscle anterior (AD) and posterior belly (PD). With a straight forceps, take the anterior tendon of the posterior belly, cut it and dissect it carefully until its origin in the tympanic bulla (ty). E: ear, Sg: submandibular gland, VII: facial nerve, M: masseter muscle, SMC: sternocleidomastoid muscle, AD: anterior belly digastric muscle, PD: posterior belly digastric muscle, Ty: Tympanic bulla.

5. Levator veli palatini muscle (derived from the 4th branchial arch)

- After dissection of the posterior belly of the digastric muscle, localize the stylohyoid muscle, pull it laterally, and carefully remove it (Figure 4A).
- Localize the tendon of the levator veli palatini that inserts at the tympanic bulla (Figure 4A). Dissect it carefully and cut it on both sides.
- Look for the trachea and the esophagus that runs behind it. Lift the esophagus, and expose the pharynx and the larynx.
- Localize and dissect the area of the superior pharyngeal constrictor muscle. Identify the levator veli palatini and cut it at both sides (Figure 4B).

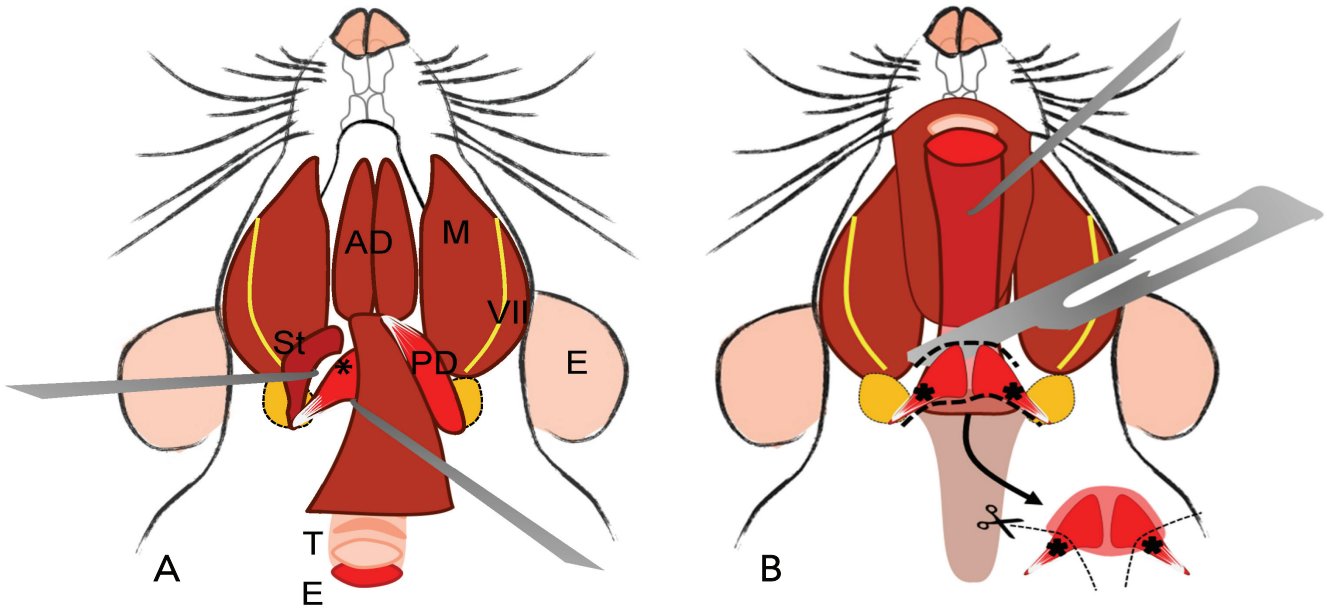


Figure 4. Dissection of the levator veli palatini muscle. (A) General view after dissection of the digastric muscle (posterior belly). Stylohyoid muscle (St) and tendon of the levator veli palatini can be localized. Note the trachea (T) and esophagus (Es) running behind it. (B) After lifting the trachea and the esophagus the pharynx (P) is exposed. The levator veli palatini that runs laterally towards the soft palate is now visible. The arrow indicates the dissected superior pharyngeal constrictor muscle; note the levator veli palatini muscles at both sides. E: ear, St: stylohyoid muscle, VII: facial nerve, M: masseter muscle, AD: anterior belly digastric muscle, PD: posterior belly digastric muscle, T: trachea, Es: esophagus, P: Pharynx, *levator veli palatini muscle.

Note: Directly after dissection, carefully remove tendon and connective tissue from each muscle under the stereo microscope. Submerge all specimens quickly in ethanol 70%, and transfer them to ice-cold PBS supplemented 2% P/S in a 15 ml tube.

Isolation of Satellite Cells

1. Perform the following preparation steps for SC isolation from 3 groups of muscles:
   -. Prepare 7.5 ml of 0.1% pronase in DMEM. Filter the solution through a 0.22 µm filter. Pre-warm the solution at 37°C in a water bath for 10 min before isolation.
   - Prepare 35 ml of DMEM supplemented with 10% Horse Serum (HS) and 1% P/S. Also pre-warm at 37°C in a water bath.
   - Prepare 15 ml culture medium which consists of DMEM supplemented with 20% fetal bovine serum (FBS), 10% HS, 1% P/S and 1% chicken embryo extract (CEE). Pre-warm at 37°C in a water bath.
   1.4. Pre-coat six plastic pipettes (10 ml) with HS and dry for at least 10 min before use.
2. In the culture hood, transfer each muscle into a well of a 6-well plate. Using the dissection scissors, cut the muscle in small pieces of about 2mm. Be careful not to mince the tissue too much.
3. Carefully add 2.5 ml of 0.1% pronase solution to each well and incubate at 37°C for 60 min. Gently shake the plate after 20, 40, and 60 min.

Note: The exact duration of the incubation depends on factors like age and strain of the animals.

4. Monitor under the microscope. Check the muscle fragments and stop the enzymatic digestion when the fibers bundles get a loosened appearance (Figure 5).

5. Add 2.5 ml of DMEM supplemented with 10% HS and 1% P/S. Transfer to a 15 ml tube and centrifuge the tubes at 400 x g for 5 min. Discard the supernatant by decantation.
6. Add 5 ml DMEM supplemented with 10% HS and 1% P/S. Pipette the solution up and down with a 10 ml plastic pipette (trituration) for at least 20 times to homogenize the tissue.

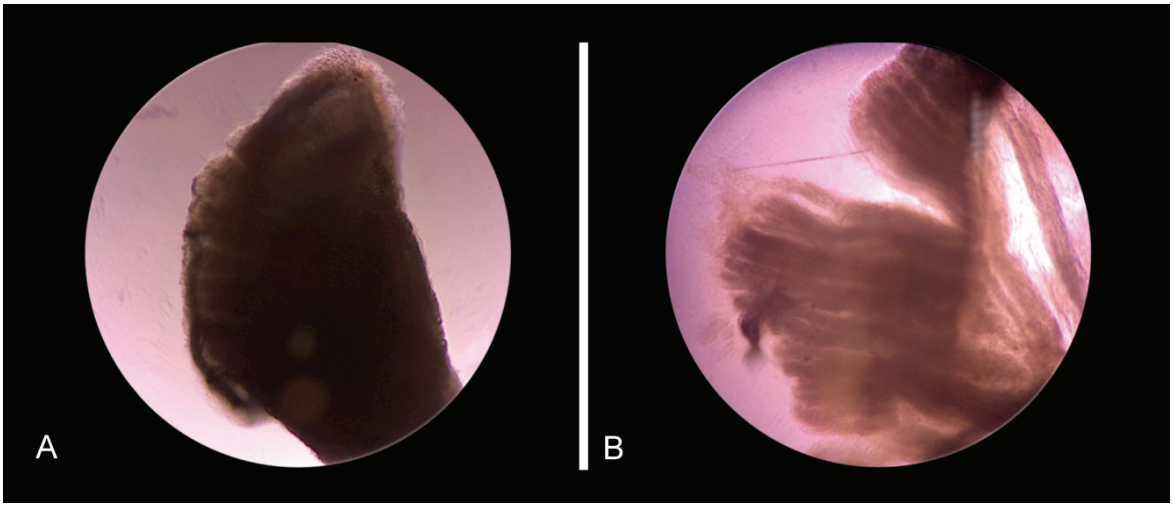


Figure 5. Appearance of the muscle tissue. (A) before and (B) after enzymatic digestion with pronase. Note that muscle bundles appear to be loosened after enzymatic digestion.

7. Centrifuge the tubes at 200 x g for 4 min. Collect the supernatant and transfer into a 15 ml tube.
8. Add 5 ml DMEM supplemented with 10% HS and 1% P/S. Pipette again with a 10 ml plastic pipette until the tissue fragments passes easily through the pipette.
9. Centrifuge the tubes at 200 x g for 4 min and collect the supernatant in a 15 ml tube.
10. Put a cell strainer (40 µm) onto a 50 ml tube and transfer the supernatant containing the dissociated cells onto the filter. Wash with 1 ml DMEM for maximal cell recovery.
11. Centrifuge the tubes at 1,000 x g for 10 min and discard the supernatant with a pipet.

12. Resuspend the pellet in 300 µl culture medium and count the cells in a hemocytometer.

Differentiation of Satellite Cells on Extracellular Matrix Gel Spots

1. Dilute the cell suspension to obtain 1.5 x 103 cells in 10 µl of culture medium.
2. Secure the covers of the chambers slides with tape and mark the spots with a black marker on the bottom side of the object glass.
3. Using a micropipette, put a drop of 10 µl cell suspension onto the extracellular matrix gel spot. Check under the microscope whether the drop of cell suspension has been placed correctly on the spot. Incubate for six hours at 37°C.
4. Carefully add 400 µl of culture medium (DMEM supplemented with 20% FBS, 10% HS, 1% P/S and 1% CEE) and incubate for three days at 37°C.

Note: At this point, freshly isolated SC are subjected to massive trauma (enzymatic digest and harsh trituration) and they need to recover. Do not disturb the cells during the first three days 37. Next, the culture medium can be changed depending on the type of experiment. The extracellular matrix gel spots can be seeded with a high cell density (1.5-2.5 x 10^3/20 µl) for differentiation assay. Culture medium (DMEM supplemented with 20% FBS, 10% HS, 1% P/S and 1% chicken embryo extract) can be replaced every third day.

Alternatively, if expansion and passing is desired follow the next steps:

– Thaw an aliquot extracellular matrix gel (500 µl) at 4°C for at least 1.5 hr. Dilute 1:10 in DMEM and follow the recommendations in point 1.1.1.
– Pre-chill a 10 ml pipette for 10 min at 4°C.

- Transfer three T75 flasks onto a cold surface (e.g. a freezer pack) for 10 min.
- Use the pre-chilled pipette to put 1 ml extracellular matrix gel into each flask. Check that the surface is covered completely. Keep the flasks on the cold surface for at least another 7 min (Figure 1A).
- Completely remove the remaining extracellular matrix gel with a 10 ml pipette, and dry the wells at 37°C for 1 hr.
- After counting, resuspend the freshly isolated SCs in 10 ml of culture medium (DMEM supplemented with 20% FBS, 10% HS, 1% P/S and 1% chicken embryo extract) and seed in the pre-coated T75 flasks.
- After three days, change the medium (and every third day) until 80% confluence is reached. For passaging, wash the T75 flasks three times with PBS. Next add 1 ml 0.25% trypsin solution and incubate for three min at 37°C. Resuspend in 9 ml of culture medium (DMEM supplemented with 20% FBS, 10% HS, 1% P/S and 1% chicken embryo extract) and centrifuge at 200 x g for 5 min. Discard the supernatant. After counting, resuspend 1 x 10^6 cells in 1,000 µl of culture medium and freeze the cells.

Representative Results

Using this protocol, the masseter muscle (one side) yields 0.8-1 x 10^6 cells, the digastric muscle (posterior belly) yields 1.5-2 x 10^5 cells, and levator veli palatini muscle yields 1-1.5 x 10^5 cells. Cell yields depend on the muscle type, strain, and age of the animal. For comparison between the three muscle groups, freshly isolated SCs were seeded at the same cell density (1.5 x 10^3/10 µl). Directly after isolation, more than 90% of the freshly isolated cells express Pax7 (Figure 6).

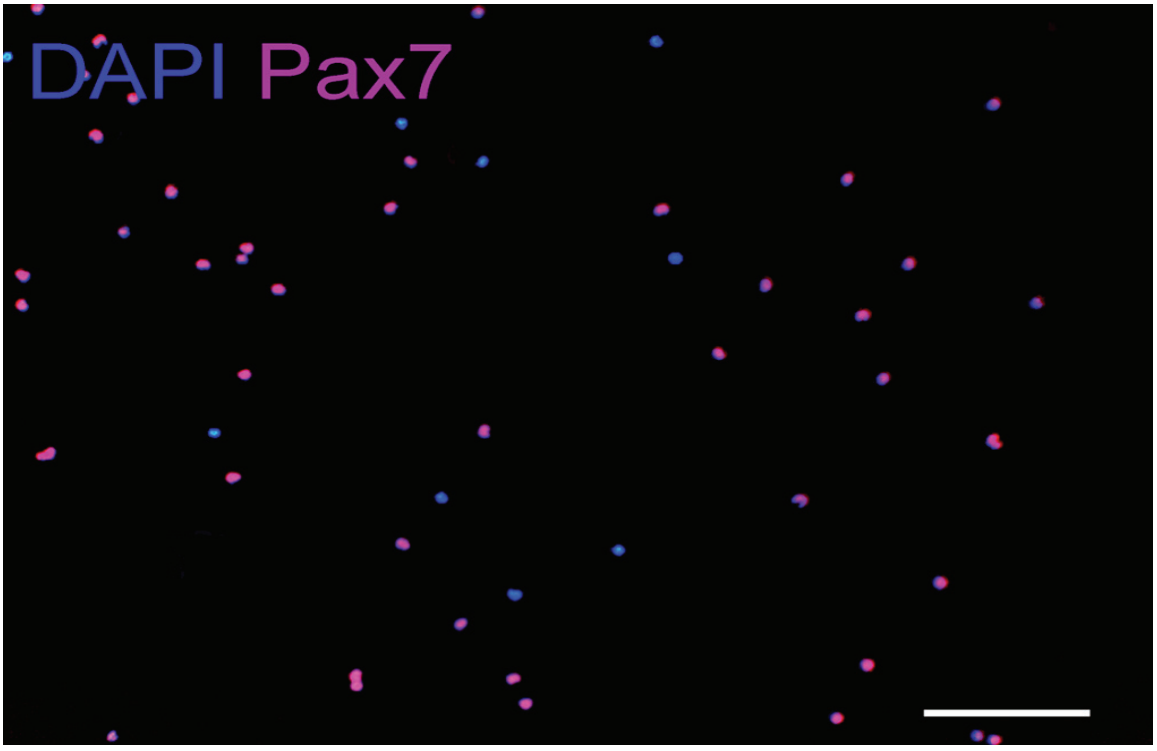


Figure 6. Pax7 immunostaining. Freshly isolated SCs, applied to extracellular matrix gel at the end of isolation (about 6 hours after initial tissue digestion). Five cells per field. Approximately 90% of the cells are Pax7 positive. DAPI: blue, Pax7: red. Scale bar, 100 µm.

Day 4, 7 and 10 cultures were stained with antibodies against Pax7, MyoD, MyoG and MyHC immunostaining. Five arbitrary fields were counted per culture using a 20X objective. At day 4 Pax7 and Myo D is expressed in all muscle groups (Figures 6 and 7 and 8), however the progeny of SCs from the masseter and digastric muscles start expressing myogenin earlier than the levator veli palatini muscle (Figure 9). At day 10, the expression of MyoG is strongly reduced in all groups (Figure 9). A few days after seeding on the extracellular matrix gel spots, the proliferating cells begin to fuse and form multi-nucleated myotubes, which express myosin heavy chain. Small myotubes are clearly visible at day 7 (Figure 10). At day 10, twitching of the myotubes can be observed (Video1).

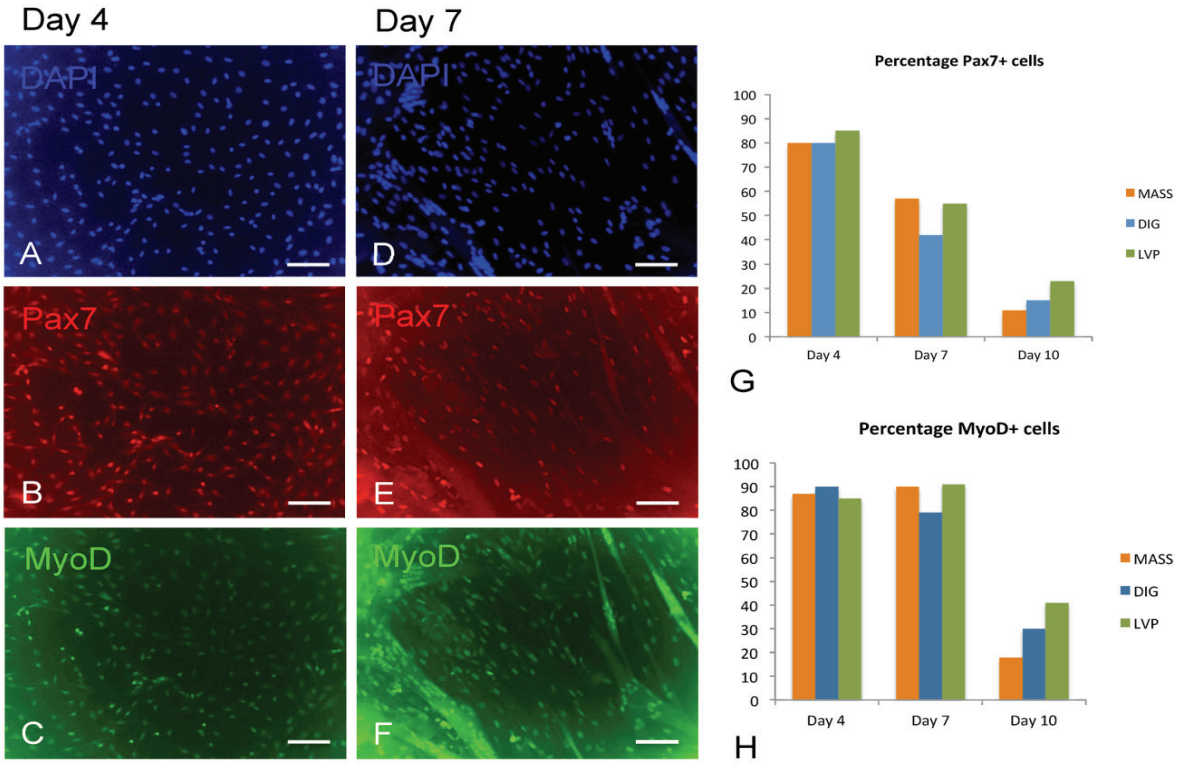
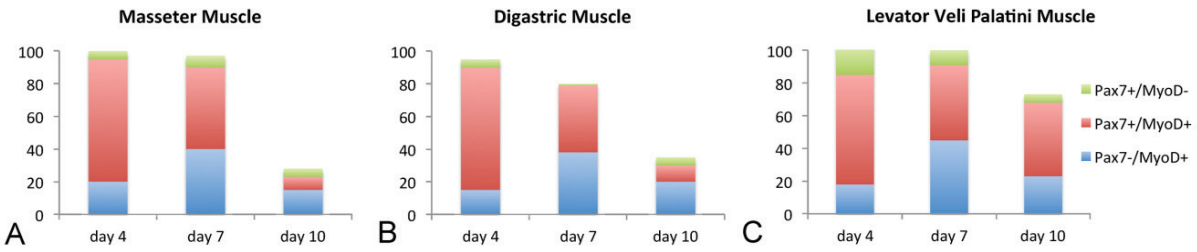


Figure 7. Pax 7, MyoD immunostaining. Day 4, 7 and 10 cultures were stained with antibodies against Pax7, and MyoD immunostaining. (A–C) and (D–F) Representative photomicrographs of day 4 and 7 cultures from the masseter muscle. (G and H) The number of Pax7+ and MyoD+ nuclei per microscopic field was counted and expressed as a percentage of the total number of nuclei (DAPI). DAPI: blue, Pax7: red, and MyoD: green. Scales bar, 100 μm.



	Days	Total number of cells	% of total cells	Pax7+/MyoD+	Pax7-/MyoD+
MASS	4	150	5	75	12
	7	337	7	50	40
	10	760	8	3	15
DIG	4	180	5	75	15
	7	551	1	41	38
	10	767	5	10	20
LVP	4	189	18	67	18
	7	405	9	46	45
	10	765	5	18	23

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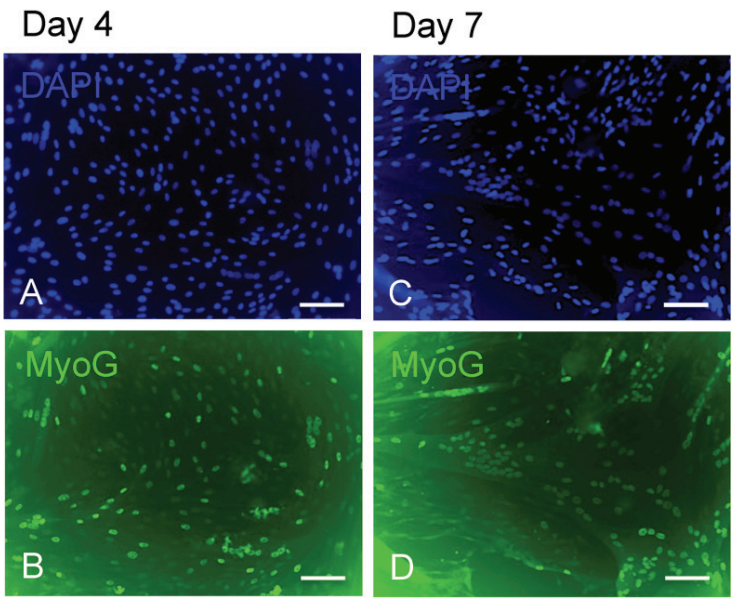
Figure 8. Distribution of Pax7±/MyoD± in cultures from mononucleated cells in cultures from masseter, digastric and levator veli palatini muscle. (A–C) Day 4, 7 and 10 cultures were stained with antibodies against Pax7, and MyoD immunostaining. The total number of cells is based on of the total number of nuclei (DAPI). (D) Data quantification of Pax7±/MyoD± cells.



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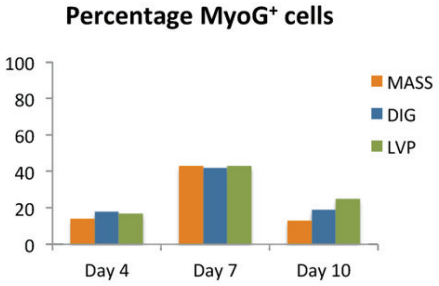

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	Days	Total number of cells	% of total MyoG+
MASS	4	180	14
	7	390	43
	10	762	13
DIG	4	183	18
	7	522	42
	10	789	19
LVP	4	192	17
	7	428	43
	10	795	25

Figure 9. Myogenin immunostaining. Day 4, 7 and 10 cultures were stained with antibodies against Myogenin. (A–D) Representative photomicrographs of day 4 and 7 cultures from the levator veli palatini muscle. (E) The number of MyoG+ nuclei per microscopic field was counted and expressed as a percentage of the total number of nuclei (DAPI). (F) Data quantification of MyoG+ cells. DAPI: blue, Myogenin: green. Scales bar, 100 μm.

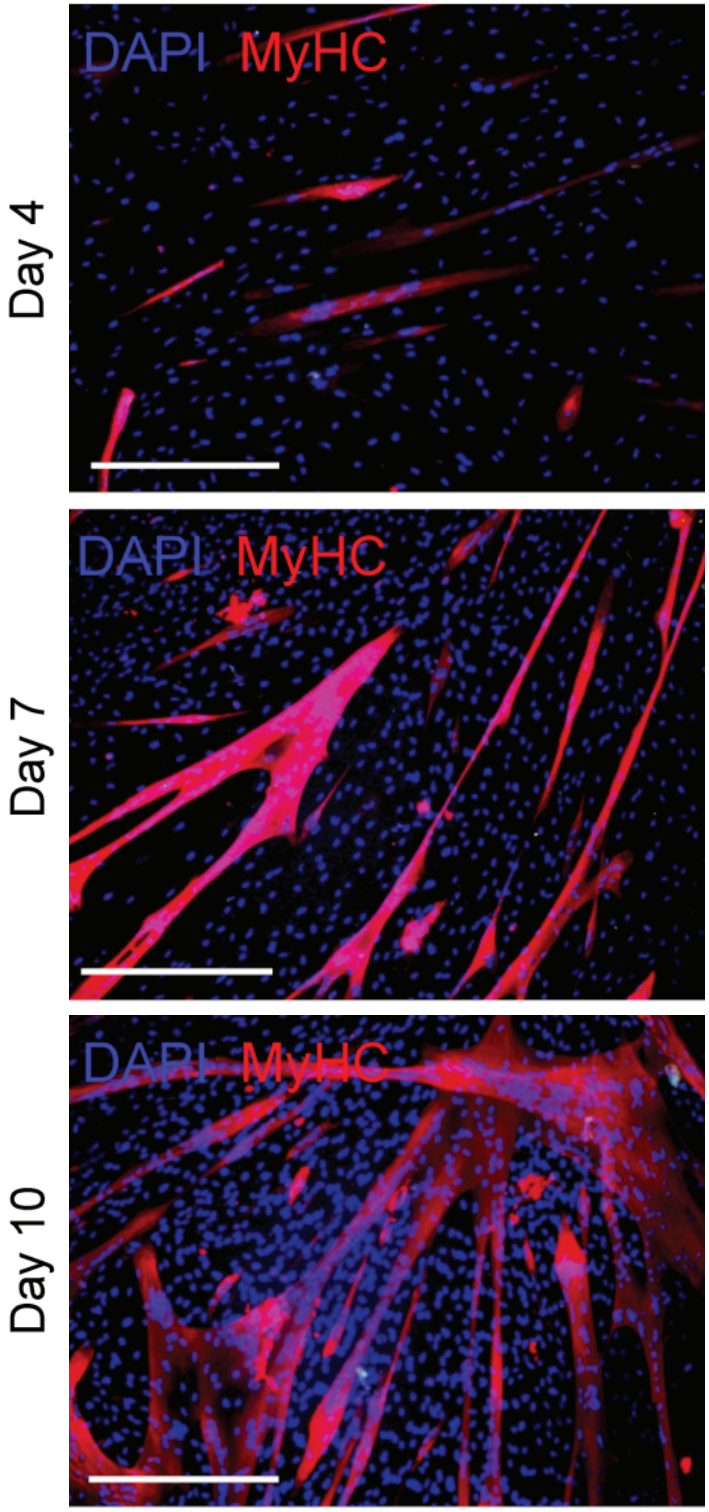

Figure 10. Myosin Heavy Chain immunostaining. Day 4, 7 and 10 cultures were stained with antibodies against myosin heavy chain (MyHC). Representative photomicrographs of day 4, 7 and 10 cultures from the digastric (DIG) muscle. At day 7, small myotubes are present while at day 10 long and well-organized myotubes are evident. Scales bar, 200 µm.

Video 1. Myotube twitching. Examples of two representative fields with twitching myotubes are shown for day 10 cultures from digastric muscle. Video link can be found here: http://www.jove.com/video/52802/isolation-characterization-satellite-cells-from-rat-head

Discussion

SCs from different branchiomeric head muscles were isolated from one 9-week-old Wistar rat and cultured directly on extracellular matrix gel spots without prior expansion and passaging. After isolation, the cells were counted and seeded at the same cell density. For the parallel isolation of three different muscles, this method takes about 4 hr. To avoid culture contamination, a critical step is the rapid washing in alcohol 70% after dissection of the muscles.

During SC isolation it is important to cut the muscle tissue into small pieces (about 2 mm) but avoid too much mincing as this will result in a small cell yield because of cell damage. Also, the duration of the enzymatic digestion must be checked carefully under the microscope to avoid further damage. The aim of the digestion is to dissociate the myofibers. Since more than 90% of the isolated cells express Pax7, no further purification is required (Figures 6-8). This avoids extra purification steps in other methods such as pre-plating on uncoated dishes,[14,37,38] fractionation on Percoll,[39,40] or fluorescent- or magnetic cell sorting.[41,43] For trituration it is essential to induce shear between the tissue fragments and the opening of the pipette tip as this allows the mechanical release of the SCs. If the trituration with a 10 ml pipette (inside diameter tip: 1 mm) is difficult, a 5 ml (inside diameter tip: 2 mm) pipette can be used first. Alternatively, glass Pasteur pipettes can be cut at the desired diameter and be used. This method is simple, efficient and allows the simultaneous isolation of SC from different muscle samples.

The culture plates for SCs can also be coated with gelatin or collagen, but our previous studies show that extracellular matrix gel is far better for the maintenance of the myogenic potential than collagen.[38] The extracellular matrix gel spots of millimeter size (10 µl/Ø 2 mm or 20 µl/Ø 4 mm) allows the study of proliferation and differentiation of SCs with limited numbers of cells. For the differentiation assay about 8 to 20 times fewer cells are required compared to a 24-well plate (Ø 15.6 mm), and about 80 to 200 times fewer compared to 35 mm Petri dishes (Ø 35 mm).[14,38]

Since extracellular matrix gel is expensive, this method is also more cost-efficient. In addition, the chamber slides can be replaced by plastic cover slips to further reduce the costs. For the preparation of the extracellular matrix gel spots overnight drying of the chamber slides is essential. As the extracellular matrix gel spots are transparent, it is necessary to mark the spots at the bottom side using back lighting. The chambers slides are fixed in a Petri dish for easy manipulation. Further cell culture expansion is not necessary, which offers the possibility to study the SCs of smaller muscles or small muscle samples. Alternatively, *e.g.* for PCR or muscle constructs if more cells are needed, the freshly isolated SCs can first be expanded in T75 flasks as indicated above.

SCs isolated using this protocol are not suitable for further purification with flow cytometry immediately after isolation. The digestion with pronase causes extensive digestion of the surface antigens.[14] Horse serum and fetal bovine serum that are used for cell culture must first be properly characterized before isolation, as different lot numbers differentially affected myoblasts proliferation and differentiation.

In recent years, there is a growing interest in the muscles derived from the branchial arches and the head mesoderm (*e.g.* the extraocular muscles).[24] It has been clearly demonstrated that head and limb muscles possess highly different properties.

Masseter muscle from old animals seems to retain their regenerative capacity in comparison with limb muscles.[25,26] SCs from the extraocular muscles possess a robust proliferation and differentiation capacity comparable to SCs from head muscles, and show a larger engraftment potential than limb muscle SCs.[24] The fiber type distribution and myosin composition varies among muscle groups and also between species. Muscles originating from the first branchial arch in humans contain both slow and fast fibers (subtypes IIA and IIX), neonatal myosins and myosins typical for developing cardiac muscle. In rodents these muscles contain about 95% fast fibers myosin IIA and IIb).[44-46] Studies on avian muscles show that SCs from different muscle fiber types vary in differentiation capacity. SCs from fast fibers only differentiate into fast muscle fibers, while SCs from slow fibers can differentiate into both fiber types.[47] In addition, the percentage of SCs in fast muscle fibers is lower than in slow muscle fibers.[48,49] This indicates that the fiber type distribution must be taken into account for studies on muscles in the craniofacial area. Similar to cleft palate muscles, the levator veli palatini muscle in rodents contains almost exclusively fast fibers.[50] For that reason, SCs from the levator veli palatini are suitable for pre-clinical studies in the field of cleft palate.

This protocol offers new possibilities to study SCs derived from branchiomeric head muscles or other smaller muscles or smaller muscles samples. This will facilitate the development of new therapies to improve the regeneration of muscles in the maxillofacial area in conditions such as cleft palate but also in other conditions affecting smaller muscles.

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CHAPTER 6

NEONATAL SATELLITE CELLS FORM SMALL MYOTUBES *IN VITRO*

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Abstract

Although palatal muscle reconstruction in patients with cleft palate takes place during early childhood, normal speech development is often not achieved. We hypothesized that the intrinsic properties of head satellite cells (SCs) and the young age contribute to the poor muscle regeneration after surgery. First, the fiber type distribution and the expression of SC markers in *ex vivo* muscle tissue from head (branchiomeric) and limb (somite-derived) muscles from neonatal (2-week-old) and young (9-week-old) rats were studied. Next, SCs isolated from these muscles were cultured for 5, 7 and 9 days. Next, we investigated the *in vitro* expression of SC markers, and calculated the proliferation, early differentiation, and fusion index (myotube formation).

Ex vivo, virtually all myofibers in both masseter and levator veli palatini muscles contained fast myosin heavy chain (MyHC), while only a small percentage of digastric and extensor digitorium longus myofibers contained slow MyHC. This was independent of age. More SCs were found in muscles from neonatal rats compared with young rats (17.6±3.8 vs 2.3±1.6%, $p<0.0001$). *In vitro*, young branchiomeric head muscle (BrHM) SCs proliferated longer and differentiated later than limb muscle SCs. No differences were found between SC cultures from the different BrHM. SC cultures from neonatal muscles showed a much higher proliferation index than those from young animals at day 5 (0.8 vs 0.2, $p<0.001$). In contrast, the fusion index in neonate SCs was about twice as low as in SCs from young muscles at day 9 (27.6±1.4 vs 62.8±10.2, $p<0.0001$).

In conclusion, SCs from BrHM differ from limb muscles especially in their delayed differentiation. SCs from neonatal muscles form myotubes less efficiently than those from young muscles. These age-dependent differences in stem cell properties urge careful consideration for future clinical application in patients with cleft palate deformity.

The soft palate is the posterior muscular extension of the hard palate and its proper functioning is vital for normal speech, swallowing, blowing and sucking. About 1:500 to 1:1,000 newborns have a cleft in the lip and/or palate, which makes this the most common congenital craniofacial malformation in humans.[1] If a cleft in the soft palate is present, the palatal musculature is disorganized and abnormally inserted into the bony palate. Hence, surgical reconstruction of the muscle sling is required to improve the function. However, velopharyngeal dysfunction persists in 7 to 30% of the treated patients and deficiencies in speech are common.[1-3] Several factors contribute to the poor outcome of surgery but fibrosis seems to be an important factor.[4, 5]

Muscle progenitors, the satellite cells (SCs) are located between the basal lamina and the plasmalemma of the myofiber.[6] Following muscle injury, these cells become activated, proliferate and then differentiate into myoblasts. Differentiating myoblasts can then fuse to form new myofibers or repair damaged ones. Quiescent SCs express the transcription factor Pax7,[7] while their progeny, the proliferating myoblasts, additionally express the myogenic determination factor 1 (MyoD).[8] Differentiating myoblasts express myogenin (MyoG).[9] The terminal differentiation of myoblasts is marked by their fusion into myofibers and the expression of muscle-specific proteins such as myosin heavy chain (MyHC).[8, 9]

Soft palate muscles originate from the branchial arches and differ from somite-derived skeletal muscles (trunk, limb, ventral pharyngeal and tongue muscles).[10] Branchiomeric head muscles (BrHM) are known to regenerate slower and develop more fibrous connective tissue after injury when compared to limb muscles.[11] We previously demonstrated that SCs in injured palatal muscle do migrate to the site of injury but fail to form new myofibers. We hypothesized that not only fibrosis but also the intrinsic properties of head SCs account for the poor regeneration of palate muscles

after injury. First, the fibre type distribution and the expression of SC markers were studied in *ex vivo* muscle tissue. Next, we investigated the *in vitro* expression of SC markers, and calculated the proliferation, early differentiation and fusion index (myotube formation) of SCs isolated from representative muscles originating from the 1st, 2nd and 4th branchial arches. These muscles were, respectively, masseter (Mass), digastric (Dig) and levator veli palatini (LVP) were included. As a reference, SCs from the somite-derived extensor digitorum longus (EDL) muscle of the limb were analyzed. Since the repair of a soft palate cleft usually takes place in early childhood age may also be an important determining factor. Hence, we performed all studies in neonatal (2-week-old) and young (9-week-old) rats.

Material and Methods

Animals

The protocol was approved by the local board for animal experiments (Dier Experimenten Commissie, DEC) from the Radboud University Nijmegen in accordance with Dutch laws and regulations (RU-DEC 2013-205). Twelve male Wistar Unilever rats (Harlan BV, Horst, The Netherlands) of 2- and 9-weeks old (neonatal and young rats respectively). were housed under standard laboratory conditions.

Experimental procedures

Dissection of the superficial head of the Mass, Dig and LVP muscles was performed in the animals as previously described.[12] For the dissection of the EDL muscle from the same animals, the skin of the lower limb of the animals was shaved and removed to expose the underlying muscles. Then, the distal tendon of the tibialis anterior and EDL was cut after which the EDL was identified and isolated by cutting the proximal tendon. All the muscles were harvested for immunohistochemistry (n=3, for each age group) or cell culture (n=3, for each age group).

Immunohistochemistry

Dissected muscles were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and processed for paraffin embedding. The specimens were sectioned transversally (Figure 1) and stained as described previously in detail.[13] The following primary antibodies were used: mouse anti-fast MyHC (1:5000; Sigma Chemical Co, Zwijndrecht, The Netherlands), mouse anti-slow MyHC (1:5000; Sigma Chemical CO), mouse anti-Pax7 (1:100; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA), mouse anti-MyoD (1:50; DAKO, Dakopatts, Glostrup, Denmark), and mouse anti-myogenin (MyoG; 1:100; DSHB).

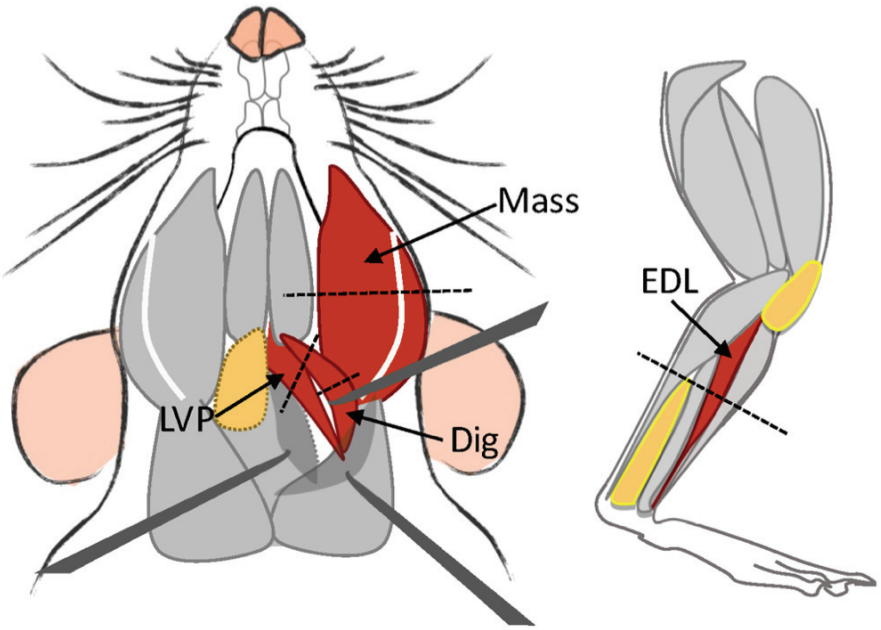

Figure 1. Schematic representation of head and limb in rats. Left: ventral view of the head. The dashed lines indicate the level of sectioning for superficial masseter (Mass), digastric (Dig) and levator veli palatini (LVP) muscles. Right: lateral view of a hind limb. The dashed line indicates the level of sectioning for the extensor digitorium longus muscle (EDL).

Satellite cell isolation

After dissection, SCs were isolated from the EDL and branchiomeric muscles (Mass, Dig and LVP) obtained from 2- and 9-weeks old rats following a protocol described previously.[12] Briefly, the tissue was

minced and treated with 0.1% Pronase for 1 h at 37°C (with gentle swirling every 20 min) followed by vigorous trituration to release cells from the muscle bulk. The cell suspensions were filtered through cell strainers (70 µm) and cells were then harvested by centrifugation at 1000×g for 10 min. Freshly isolated cells were incubated in tissue culture flasks coated with 1 mg/mL Matrigel and cultured for three days at 37°C. At day 4, cells were trypsinized and used for further culture experiments. This method of isolation yields > 90% of Pax7-positive cells at day 0.[12]

Cell culture and differentiation of satellite cells

As described previously,[12] for each muscle group, SCs were seeded at the same cell density (2 x 10^3/20µL) on Matrigel spots (1mg/ml) in chamber slides. Next, culture medium (Dulbecco's modified Eagle's medium with 4,500 mg/L glucose, 4 mM L-glutamine, and 110 mg/ml sodium pyruvate supplemented with 20% fetal bovine serum, 10% horse serum, 1% penicillin-streptomycin and 1% chicken embryo extract) was added. Next, cell cultures were incubated at 37°C in a humidified tissue culture incubator. Medium was replaced every other day. At days 5, 7, and 9 after seeding, cells were washed with DMEM and fixed in 2% formaldehyde for 10 min.

Immunofluorescense staining of satellite cell cultures

Fixed cell cultures were washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min. The cells were washed again with PBS and then incubated in blocking buffer containing 1% of normal goat serum for at least 24 hours. After washing with TRIS-buffered saline, cells were incubated with a mouse anti-Pax7 (1:50; DSHB, Iowa City, CA, USA), mouse anti-MyoG (F5D, 1:200; DSHB), rabbit monoclonal anti-MyoD1 (EP212, 1:100, Cell Marque) or mouse monoclonal anti-MyHC (MF20, 1:500). Secondary antibodies used were goat-anti-mouse IgG Alexa Fluor 594 and goat-anti-rabbit IgG Alexa Fluor 488 (Both Invitrogen, 1:1000 dilution).

Quantification

For the analysis of immunohistochemical sections and immunofluorescence cell staining ImageJ[14] was employed to facilitate manual counting of type I and II muscle fibers or cells within 9 muscle cross-sections from 3 different rats. Muscle cross-sections contained at least 400 to 500 muscle fibers and/or cells (10x magnification). For the analysis of primary cell cultures, five to ten arbitrary fields of each culture were counted at 10x or 20x magnification, depending on overall cell density at the time point of interest (a minimum of 200 cells were counted). For each cell marker, nine wells from three independent experiments were quantified. The data are expressed as the mean percentage ± SD of the total number of nuclei as indicated in the figure legends. For de index ratios, we used automatic counting of double stained pictures with ImageJ. The proliferation index was defined as the number of nuclei positive for both Pax 7 and MyoD (Pax7+ and MyoD+) divided by the total of Pax7+ cells. The early differentiation index was defined as the number of nuclei positive for both MyoD and MyoG (MyoD+ and MyoG+) divided by the total of MyoD+ cells. The fusion index was defined as the number of nuclei within myotubes expressed as the percentage of total nuclei present in each image.

Statistics

All date was analyzed by one- or two-way ANOVA with Sidak or Tukey post-hoc test using GraphPad Prism version 6.00 for MacXOS (GraphPad Software, San Diego, CA, USA, www.graphpad.com). For all experiments, unless otherwise noted, p-values of less than 0.05 were considered significant.

Results

Immunohistochemical analysis of muscle tissue

Fiber type composition

Mass, Dig, LVP and EDL tissue sections from neonatal and young animals were stained for slow and fast MyHC (Figure 2). Mass and LVP muscles contained only fast myofibers. Although the majority of myofibers in Dig and EDL muscles were fast myofibers, also a small portion of slow myofibers could be observed (5.8±1.1% and 2.8±0.6% respectively). No significant difference in the number of slow myofibers between neonatal and young animals were found.

Pax7, MyoD and MyoG-positive cells *ex vivo*

Muscle sections were stained with antibodies against Pax7, MyoD and MyoG. In general, more Pax7+ cells were found in muscles from neonatal rats compared with young rats (17.6±3.8 vs 2.3±1.6%, $p<0.001$). Mass and LVP muscles contain significantly larger number of Pax7+ (Figure 3 and 5A) MyoD+ and MyoG+ cells than limb muscles in both neonatal and young animals. In neonates, the percentage of Pax7+ cells in Mass (20.5±4.5%) and LVP (21.3±7.4%) was higher than in both Dig (13.9±2.3%) and EDL (14,9±3,4%) ($p<0.05$). Also in young animals, the percentage of Pax7+ cells in Mass (3.9±0.7%) and LVP (3.5±0.8%) was significantly higher than in Dig (1.0±0.4%) and EDL (0.7±0.1%) ($p<0.05$, Figure 3 and 5A). Similarly, in both neonates and young animals, the percentage of MyoD+ cells was higher in Mass and LVP as compared to both Dig and EDL ($p<0.05$, Figure 4 left and 5B). Finally, in neonates the percentage of MyoG+ cells in Mass (14.4±5.2%) and LVP (14.9±3.0%) were higher than in both Dig (9.81±2.2%) and EDL (Figure 4 right and 5C). All neonatal muscles contained significantly larger number of Pax7+, MyoD+, and MyoG+ cells compared with young muscles ($p<0.0001$).

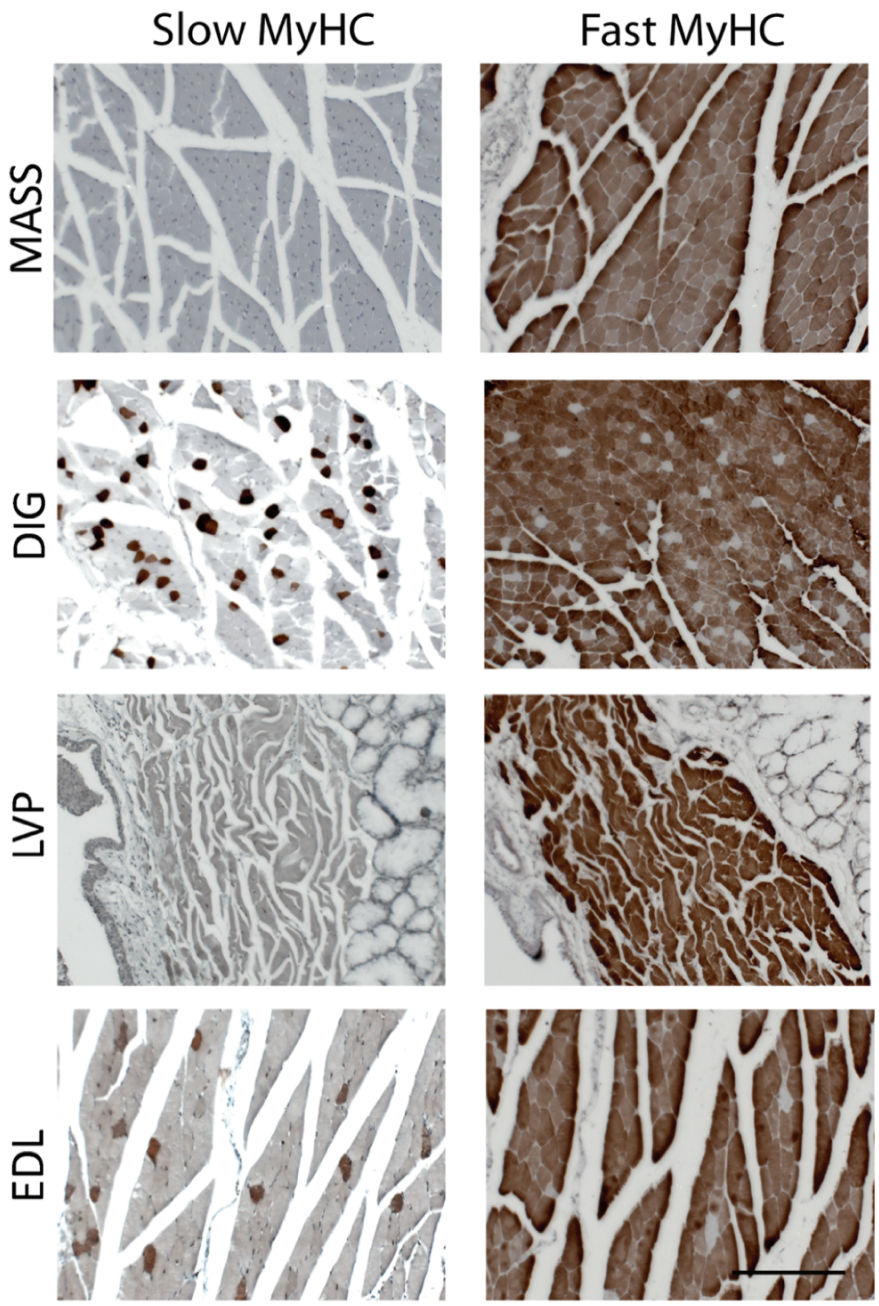

Figure 2. Fast and slow myosin heavy chain staining of muscles. Sections from the masseter (MASS), digastric (DIG), levator veli palatini (LVP), and extensor digitorium longus (EDL) muscles were stained with antibodies against slow and fast myosin heavy chain. Representative tissue sections of young animals are shown. Dig and EDL muscles contain mainly fast myofibers, and a small percentage of slow MyHC. In MASS and LVP muscles slow myofibers are virtually absent. No differences were found between neonatal and young muscles. Note that the proportion of each fiber type, however, varies between muscles and even in different regions of the same muscle, which reflects its functional demands. Scale bar, 200 μm.

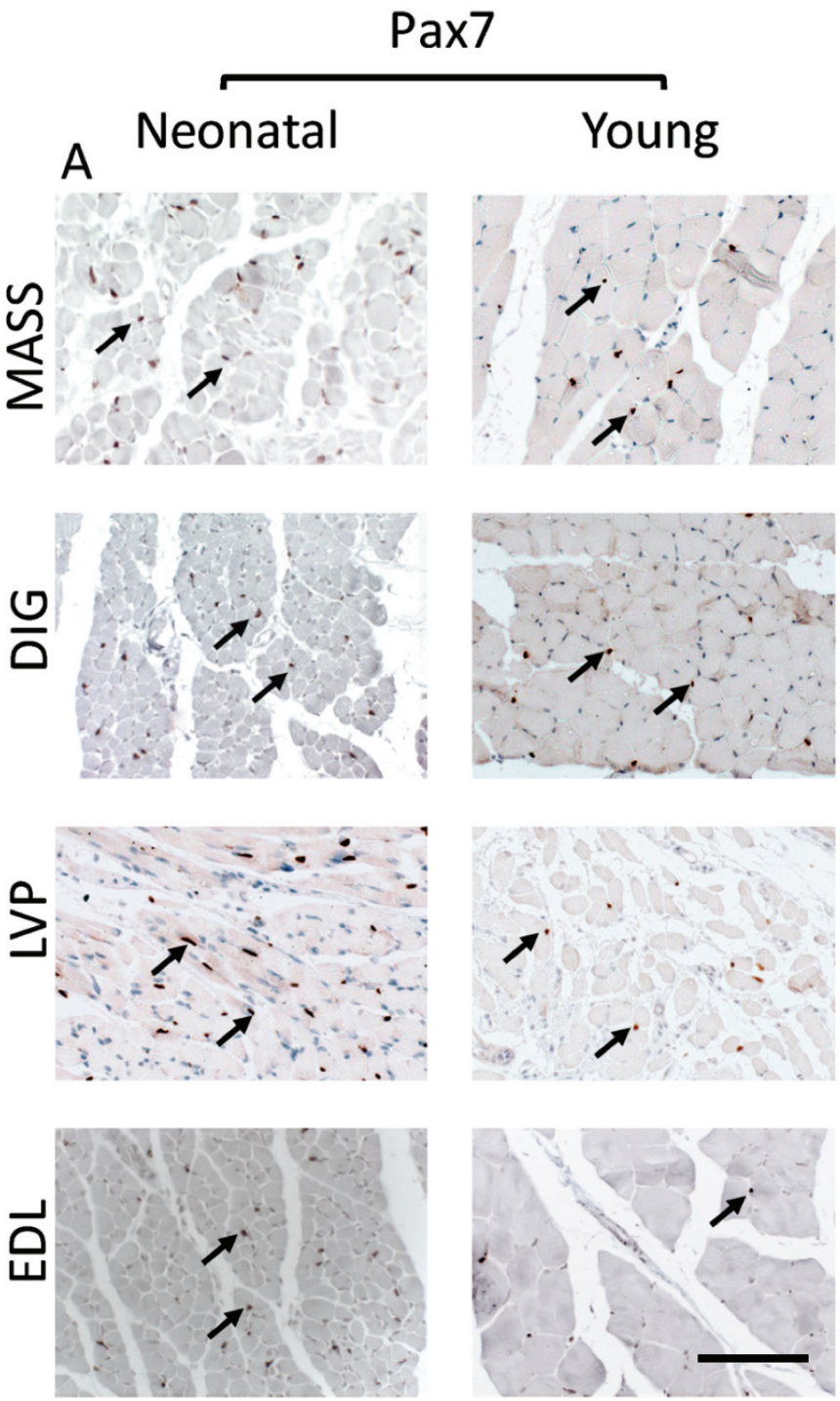

Figure 3. Pax7 positive cells *ex vivo*. Sections from the superficial head of the masseter (MASS), digastric (DIG), levator veli palatini (LVP), and extensor digitorium longus (EDL) muscles were stained with antibodies against Pax7 (positive cells in brown). Representative sections of neonatal and young muscles are shown. Note that the number of satellite cells (cells in brown) is higher in Mass and LVP than in DIG and EDL in both neonatal and young muscles. Scale bar, 100 μm.

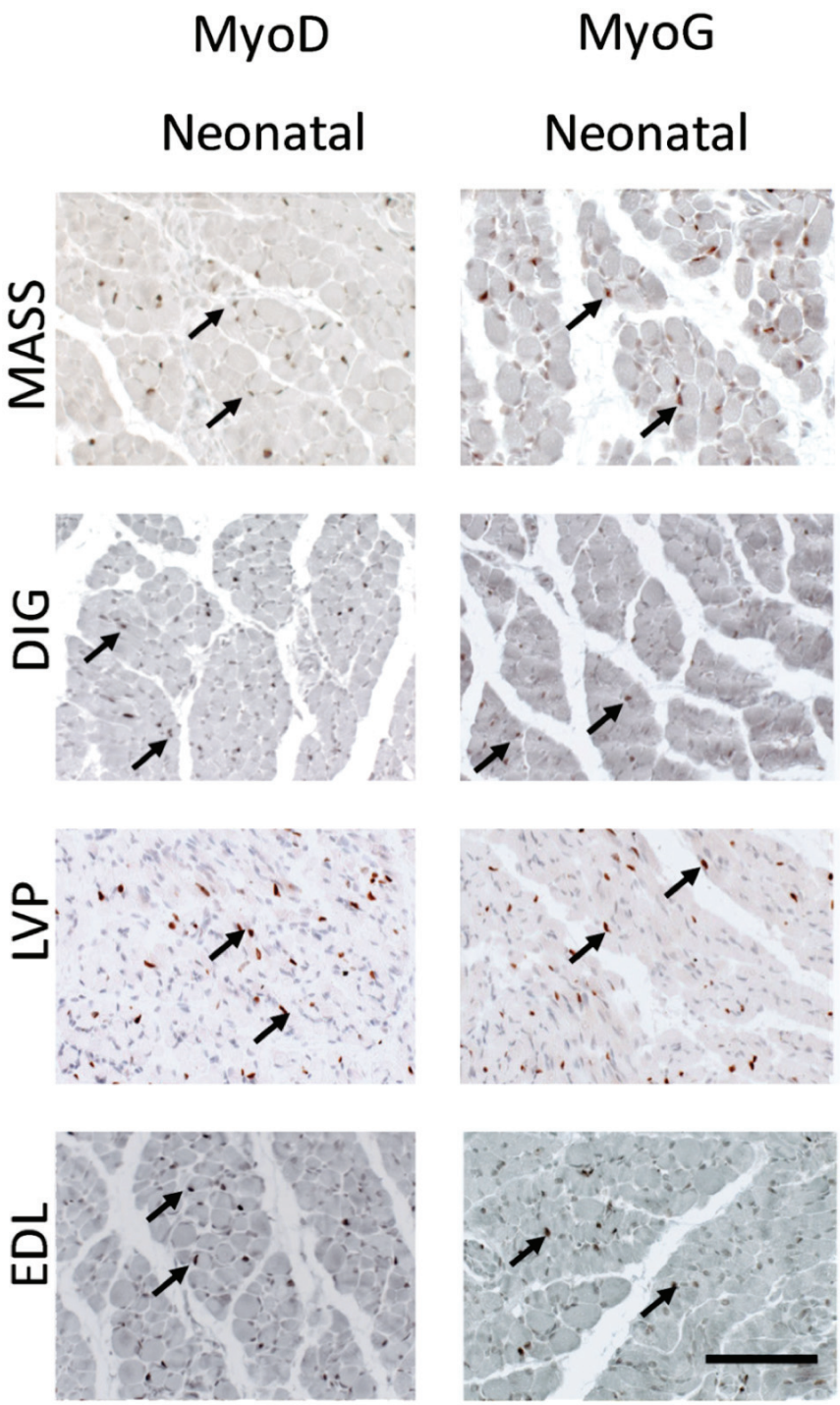

Figure 4. MyoD and MyoG positive cells *ex vivo*. Sections from the superficial head of the masseter (MASS), digastric (DIG), levator veli palatini (LVP), and extensor digitorium longus (EDL) muscles were stained with antibodies against MyoD and MyoG (positive cells in brown). Only representative sections of neonatal muscles are shown. Note that the number of of MyoD and MyoG positive cells are higher in MASS and LVP muscles. Scale bar, 100μm.

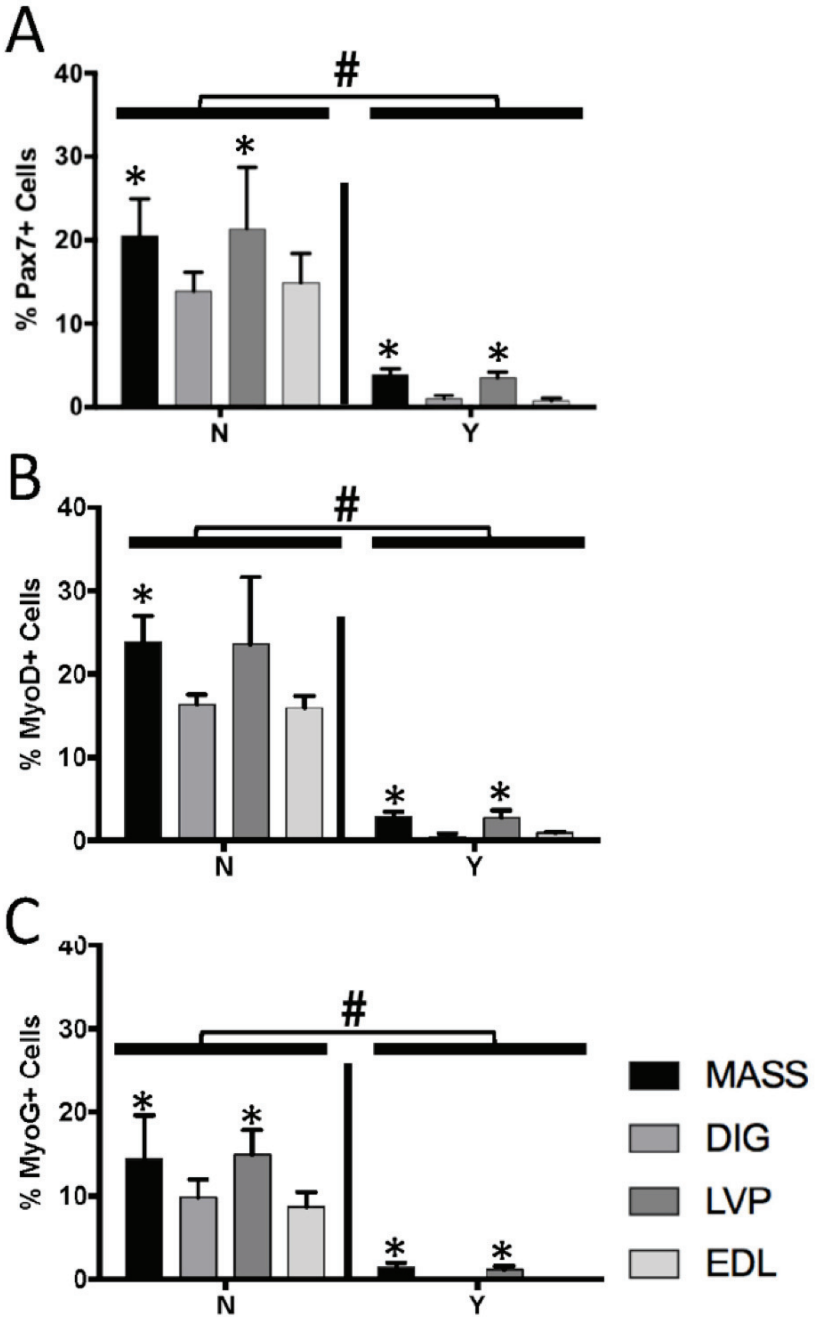

Figure 5. Pax7, MyoD and MyoG positive cells quantification *ex vivo*. The percentage of cells positive for Pax7 (A), MyoD (B), and MyoG (C) was determined as a percentage of the total number of nuclei per microscopic field. (expressed as mean ± SD). N: neonatal, Y: young. *Significant difference between muscles ($p< 0.05$); #significant difference between young and neonatal animals ($p< 0.0001$).

Pax7, MyoD and MyoG-positive cells *in vitro*

Isolated SCs from Mass, Dig, LVP and EDL from both neonatal and young animals were cultured on Matrigel spots. The primary cell cultures were fixed on days 5, 7 and 9 after isolation, and stained with markers for SCs (Pax7, MyoD, and MyoG) and MyHC. Subsequently, the percentage of Pax7+, MyoD+, MyoG+ cells was calculated. Next the proliferation, early differentiation, and fusion index was determined.

Pax7-positive cells

In cell cultures from neonates, a higher percentage of Pax7+ cells was found in Mass, Dig and LVP (92.0±1,6%, 90.9±4.1%, and 90.1±2.6% respectively) at day 5 compared with EDL cell cultures (74.6±2.1%, $p<0.05$, Figure 6A). At day 7, the number of Pax7+ cells in all cultures had significantly reduced by more than half. The percentage of Pax7+ cells in these cultures was still higher than in EDL (8.7±1.2, $p<0.05$, Figure 6A). Only few SCs were present in all cultures at day 9. The data for young animals are shown in Figure 6B. About 40% of the cells were Pax7+ at day 5. At day 7, this percentage was maintained in Mass, Dig, and LVP (43.6±2.0, 43.2±4.1, and 47.5±3.6 respectively) cell cultures but not in the EDL (17.1±4.6%, $p<0.05$). Only few Pax7+ cells were present at day 9. All cell cultures from neonates showed a higher percentage of Pax7+ cells than cultures from young animals at day 5 ($p<0.0001$).

MyoD-positive cells

In cell cultures from neonates, a higher percentage of MyoD+ cells was found in Mass, Dig and LVP (62.2±7.2%, 58.9±6.3%, and 60.7±7.0% respectively) at day 5 compared with EDL (42.7±2.4%, $p<0.05$, Figure 6C). At day 7, the number of MyoD+ cells was significantly reduced by about half. The percentage of MyoD+ cells in Mass, Dig, and LVP cell cultures (32.8±4.5%, 26.2±3.6%, 31.5±1.5% respectively) was still higher than in EDL cell cultures (11.2±1.5%, $p<0.05$, Figure 6C). Only few MyoD+ cells were

present at day 9. For young animals, the data are shown in Figure 6D. About 40% of the cells were MyoD+ cells at day 5. This percentage was maintained in Mass, Dig, and LVP (48.5±9.2, 43.7±2.1, 47.1±3.0 respectively) at day 7 but not in the EDL cell culture which was significantly decreased (28.3±8.0%, $p<0.05$). Only few MyoD+ cells were found at day 9. As the case for Pax7+ cells, all cell cultures from neonates showed a higher percentage of MyoD+ cells than cultures from young animals at day 5 ($p<0.0001$).

MyoG-positive cells

Overall, a small percentage of MyoG+ cells was present in cell cultures from neonates at days 5, 7, and 9 (Figure 6E). The highest percentage of MyoG+ cells was found at day 7 ($p<0.05$). The percentage of MyoG+ cells for young animals is shown in Figure 6F. A small percentage of MyoG+ cells were found at day 5. In contrast, about 40% of the cells MyoG+ cells at day 7. A higher percentage of MyoG+ cells was found in cell cultures from EDL (54.9±7.1%) at day 7 compared with Mass, Dig, and LVP cell cultures (39.6±6.4%, 38.8±8.9%, 38.1±7.2% respectively, $p<0.05$). No significant differences were found at day 9. Few number of MyoG+ cells were found at day 9. Cell cultures from young animals showed a higher percentage of MyoG+ cells than those from neonatal animals at day 7 ($p<0.0001$).

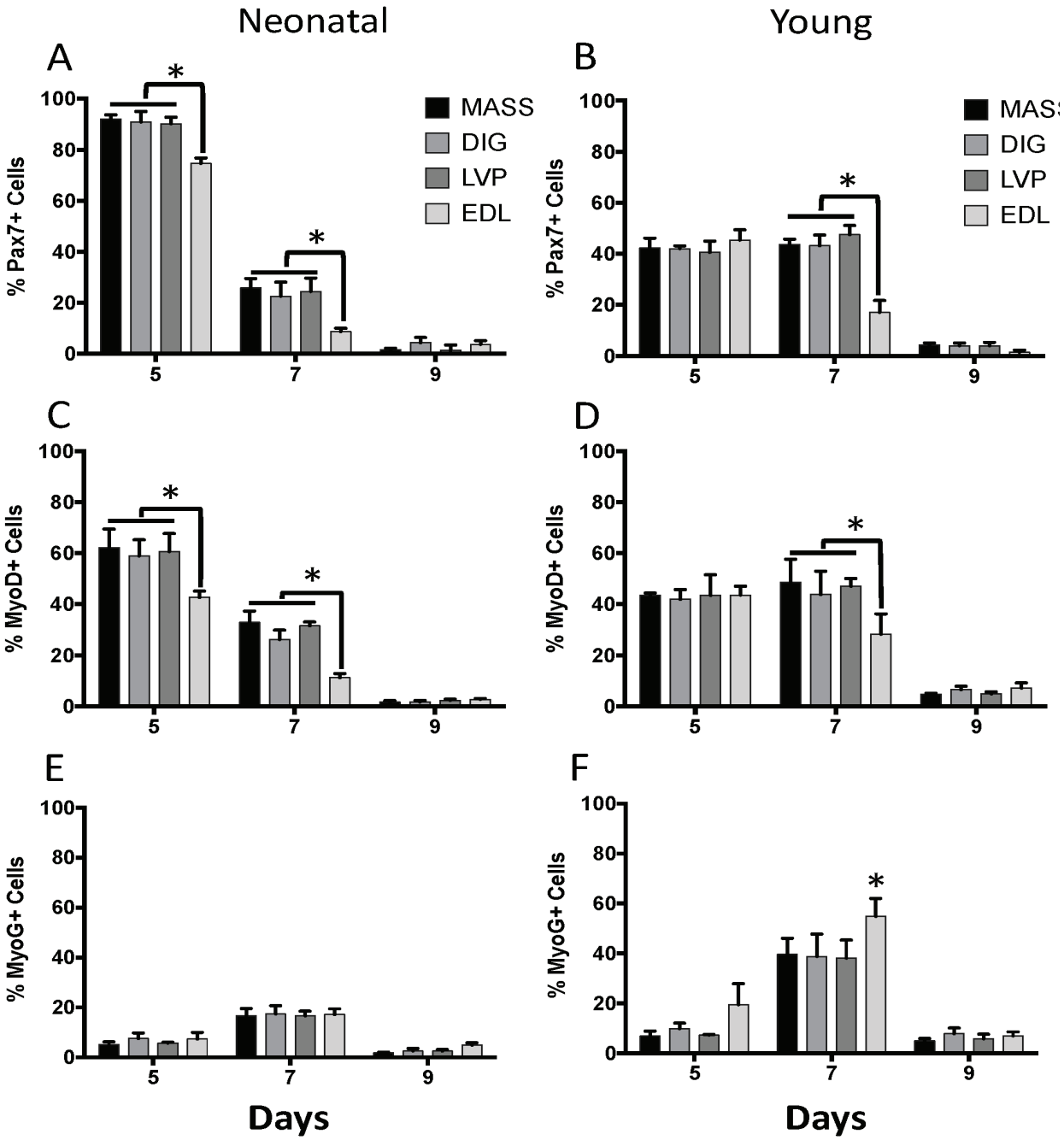

Figure 6. Satellite cell staining *in vitro*. Day 5, 7 and 9 cell cultures were stained with antibodies against Pax7, MyoD and MyoG. The number of Pax7+ (A, B) MyoD+ (C, D) and MyoG+ (E, F) nuclei per microscopic field was counted and expressed as a percentage of the total number of nuclei (DAPI+). N: neonatal, Y: young. The effect on time was significant between days 5 and 7 in A, C, E and F, between days 7 and 9 ($p<0.05$) in all groups, and between days 5 and 9 in A, B, C, D, E and F only for the head muscles. *Significant difference between muscle groups ($p< 0.05$).

Proliferation index

Cell culture from neonates and young animals were co-stained with antibodies against Pax7 and MyoD to determinate the proliferation index. The proliferation index (double positive cells divided by total Pax7+ cells) for both neonatal and young cell cultures are shown in figure 7A and 7B. In cell cultures from neonates, a higher index was found in all muscles at day 5 (0.8±0.1 Vs 0,2±0.1). This index had decreased by more than half at day 7. The proliferation index was very low at day 9 (Figure 7A). There were no differences between the muscle groups. In contrast, the index for all young cell cultures, was about 0.3 at day 5. This was maintained in all muscles except by the EDL at day 7, which shows a significant decrease (0.096±0.083, $p<0.05$, Figure 7B). The proliferation index was low at day 9. All cell cultures from neonates showed a higher proliferation index than cultures from young animals at day 5 ($p<0.0001$).

Early differentiation index

Cell culture from neonates and young animals were co-stained with antibodies against MyoD and MyoG to determinate the differentiation index. The differentiation index (double positive cells divided by total MyoD+ cells) for both neonatal and young cell cultures are shown in figure 7C and 7D. Overall, the index was low in cell cultures from neonates at days 5, 7, and 9 (Figure 7C). In young cells cultures, an index of about 0.3 was found at day 5 and 7. There were no significant differences between the muscle groups. The index was very low at day 9 (Figure 7D). Cell cultures from young animals showed a higher index than those from neonatal cell cultures at all time points ($p<0.0001$).

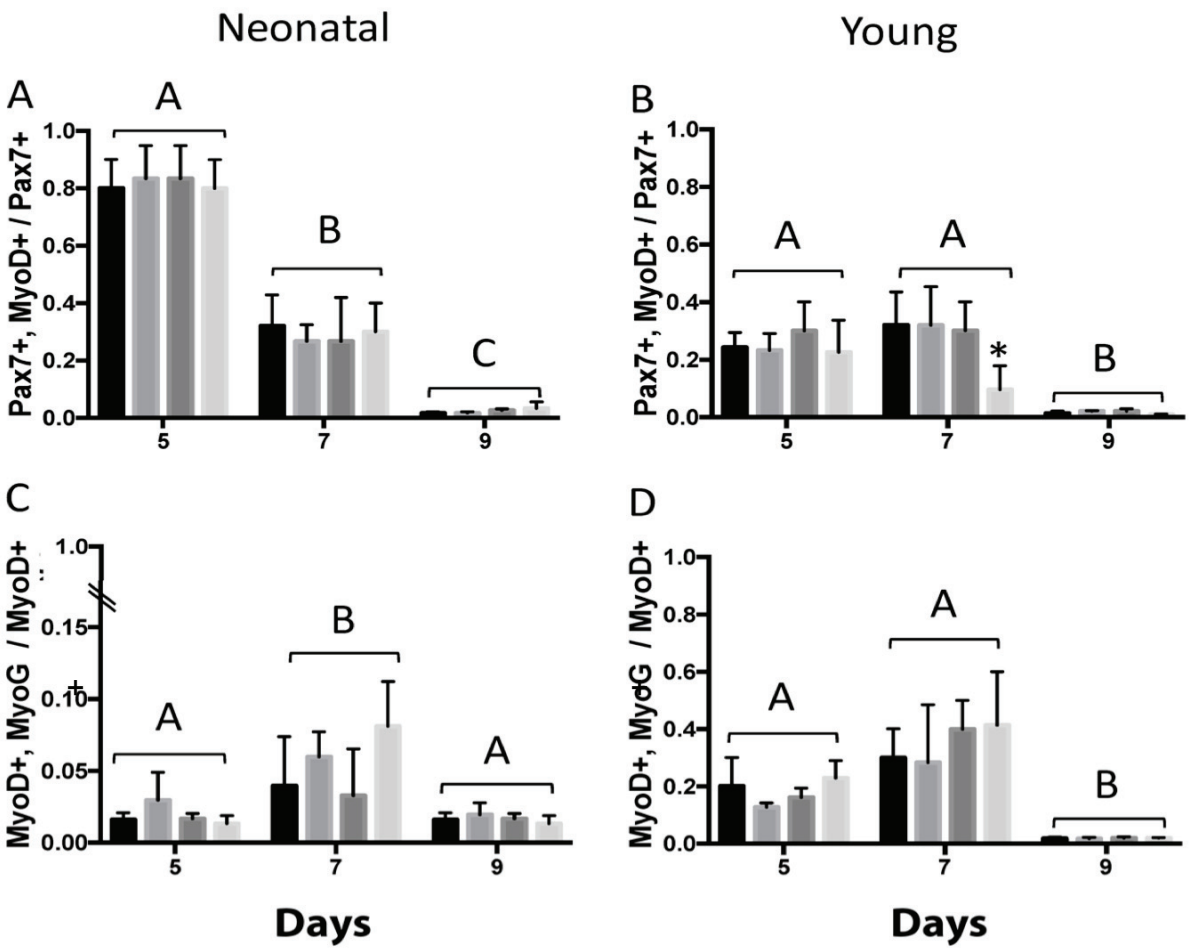

Figure 7. Proliferation and early differentiation index *in vitro*. (A and B) Day 5, 7 and 9 cell cultures were co-stained with antibodies against Pax7 and MyoD. The proliferation index was defined as the number of double positive cells divided by the total of Pax7+ cells. (C and D) Day 5, 7 and 9 cell cultures were co-stained with antibodies against MyoD and MyoG. The early differentiation index was defined as the number of double positive cells divided by the total of MyoD+ cells. *significant difference between the muscle groups ($p < 0.05$). Differences between the time points are indicated by letters ($p<0.0001$); groups with the same letter are not significantly different, groups with a different letter are significantly different.

Myotube formation

To evaluate myotube formation, cell cultures were stained with antibodies against fast MyHC. To evaluate myotube formation, cell cultures were stained with antibodies against fast MyHC. The number of fused nuclei in myotubes is expressed as fusion index in figure 8. In general, all SC cultures from both neonatal and young animals showed myotube formation at day 7. In neonates, no differences were found at any time point (Figure 8B). In young animals, no significant differences were found at day 5. EDL cell cultures (40.8±6.0%) showed a higher fusion index compared with Mass, Dig and LVP at day 7 (24.0±4.5%, 22.7±3.5%, and 25.6±5.2% respectively, $p<0.05$). In contrast, the fusion index from Mass, Dig, and LVP (70.6±3.7%, 65.8±8.5%, and 67.0±10.4% respectively) was higher than from EDL cell cultures at day 9 (47.8±7.9%, $p<0.05$, Figure 8B). The fusion index in cells from young animals was about twice as high ($P<0.0001$) compared with cells from neonates.

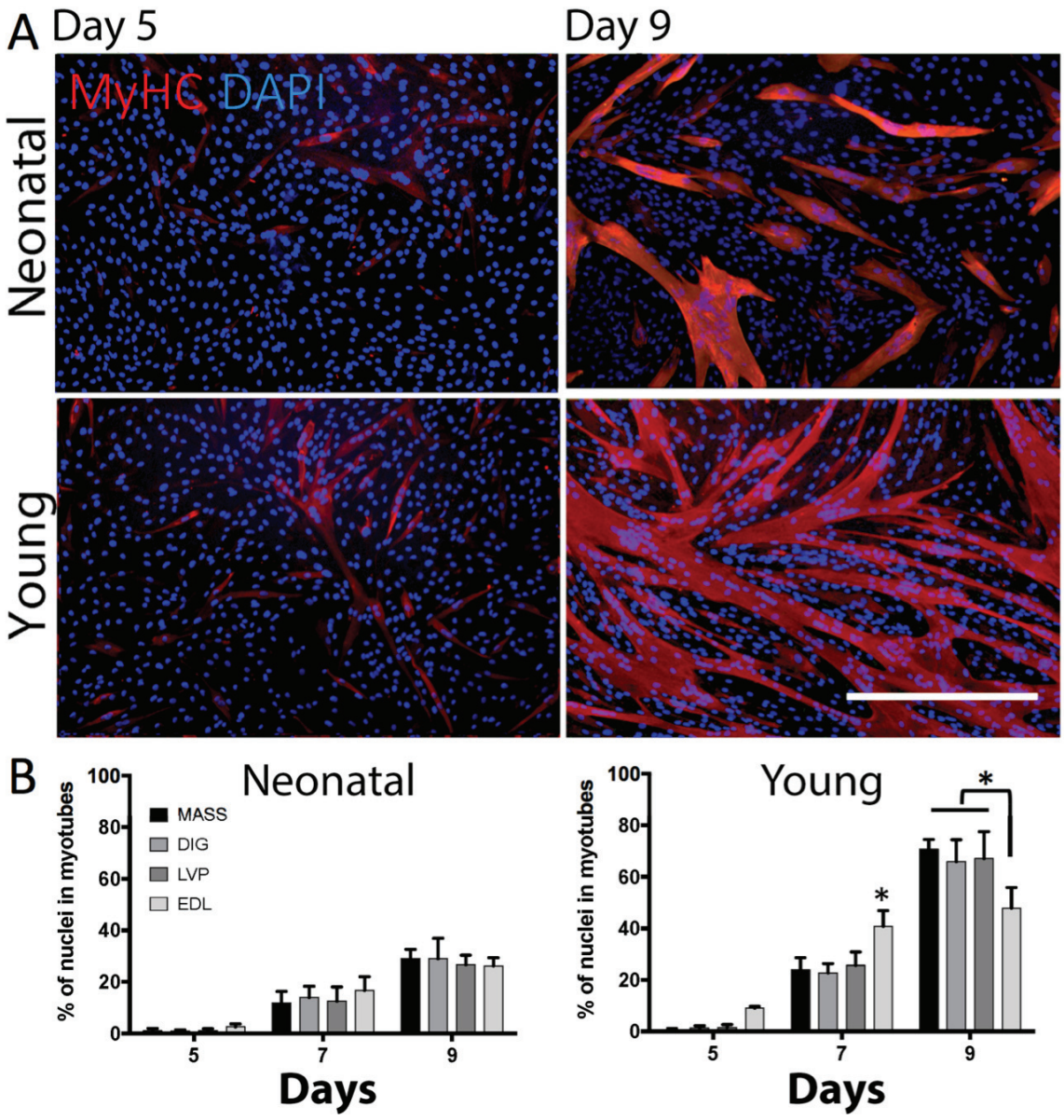

Figure 8. Myosin heavy chain staining in neonatal and young satellite *cell in vitro.* Day 5, 7 and 9 cell cultures were stained with antibodies against myosin heavy chain. (A) Representative microscopic fields of day 5 and 9 cultures from the masseter muscles from neonates and young animals. Scale bar,200 μm. (B) Fusion Index. The fusion index is determined as the percentage of nuclei lying inside the myotubes compared to the total number of nuclei per microscopic field (expressed as mean ± SD). The effect of time was significant between days 5, 7, and 9 in all groups *Significant difference ($p < 0.05$).

Discussion

In a previous study, we demonstrated that muscle regeneration in the soft palate of the rat after excisional injury is incomplete.[5] Extensive fibrotic tissue developed in the wound area with little or no formation of new myofibers. SCs did proliferate and migrate into the wound area, but failed to form new myofibers probably because large amounts of collagen had been deposited. The present study aimed to determine whether also the intrinsic properties of BrHM SCs contribute to impaired muscle regeneration in the soft palate. We compared the *in vitro* capacity for differentiation and myotube formation of BrHM SCs and limb muscle SCs in neonatal and young animals. Representative muscles originating from the 1st (Mass), 2nd (Dig), and 4th (LVP) branchial arches are compared.

The fiber type distribution and myosin composition varies between muscles and even in different regions of the same muscle, which reflects their functional demands. Muscles originating from the first branchial arch in humans contain both slow and fast fibers, neonatal myosins and myosins typical for developing cardiac muscle. In rodents these muscles contain about 95% fast fibers,[15,16] as is also the case for all muscles included in this study. The fiber type composition of head and limb muscles in our study is comparable. In addition, the percentage of SCs in fast muscle fibers is lower than in slow muscle fibers.[17] Hence, the *ex vivo* and *in vitro* differences found in our study might be related to embryological origin rather than fiber type composition. Similar to cleft palate muscles in humans, the LVP in rodents contains almost exclusively fast fibers.[13] Myoblasts from slow muscles do not express slow MyHC when there is no innervations such as during cell culture.[18]

We demonstrated that the number of SCs in Mass and LVP is higher than in EDL muscles in both neonatal and young animals. A higher number of SCs has also been demonstrated in other BrHMs from the rat such as the pharyngeal muscles compared with somite-derived muscles.[19] Also in humans, a higher number of SCs in young

adult Mass compared with young adult biceps has been reported.[20] The higher SC density observed in BrHMs seems to represent the higher turnover rate of these muscles compared with limb muscles under basal conditions.[19, 21, 22] A continuous cycle of injury and regeneration, as shown for the laryngeal muscle in humans, could be responsible for the high SC density in these muscles.[23]

As expected, neonatal muscles show a higher percentage of proliferating SCs compared with young muscles. This suggests that the SCs from muscles from young rats are largely quiescent because the demands for muscle growth are lower than in neonatal animals. In rodents, SCs comprise approximately 35% of all muscle nuclei at birth, which decreases to 10% at 4 weeks of age and less than 5% at sexual maturity when the cells are largely mitotically quiescent.[24,25] Many of these SCs are proliferating (about 80%) in growing muscle until a population of quiescent SCs is established in mature muscle.[25]

In general, there were no differences in the proliferation and early differentiation index between the SCs derived from the different branchial muscles. We did show that BrHM SCs proliferate longer in culture than SCs from limb muscles. EDL SCs isolated from young muscles seems starting to differentiate earlier than BrHM SCs resulting in a higher fusion index at early time points. However, if BrHM SCs are cultured for a longer time they exhibit a higher fusion index than EDL SCs. This was also reported by others.[26, 27] The longer proliferation phase of BrHM SCs might allow the deposition of large amounts of extracellular matrix components by fibroblasts in *vivo*. This could explain the decreased myogenic potential of BrHM SCs during healing.[28]

It is particularly interesting that SCs from neonates only form few myotubes in spite of the initially high percentage of proliferating SCs. In the neonatal growing muscle approximately 80% of SCs compose a highly proliferative population, whereas the remaining cells hardly divide.[25] *In vitro* and *in vivo* studies demonstrate that

these high proliferative SCs exclusively fuse with already existing myofibers, while the low proliferative SCs mainly fuse together.[29] This may explain the low fusion index of neonatal SCs in our experiments. Ample evidence suggests that growing immature muscles have a lower capacity for regeneration than older muscles. For instance, denervation-induced injuries are much more devastating in neonatal limb muscles than in adult muscles.[30] Also, neonatal limb SCs seem to engraft less efficiently than their adult counterparts.[31] This might be caused by the large highly proliferating fraction of SCs that is not able to participate in muscle repair. The low regenerative capacity of immature growing muscles might also contribute to the poor outcome after repair of a soft palate cleft.

Muscle regeneration and soft palate function would probably be better if surgical closure was performed in older children. However, speech and language development already start early in human life, which requires soft palate repair to be performed as early as possible.[32, 33] Thus, new adjuvant therapies that improve differentiation, fusion and myotube formation of neonatal SCs from immature clefted muscles are crucial to improve muscle regeneration and soft palate repair. Thus, new adjuvant therapies that improve differentiation, fusion and myotube formation of neonatal SCs from immature clefted muscles are crucial to improve muscle regeneration and soft palate repair.

As mentioned, no *in vitro* differences were observed between the SCs from different BrHMs. However, Mass en LVP seem to have a larger population of Pax7+ cells in both neonatal and young animals. For clinical application it is important to realize that SCs from the Mass are much easier to harvest than from the LVP. This is due to the superficial localization and simple dissection of the Mass. Also, the Mass muscle can be accessed intraorally for harvesting, which does not yield a visible scar. Thus, the Mass muscle seems to be an ideal SC source for cell–based therapy in cleft lip and/ or palate repair and other craniofacial muscle reconstructions.

In summary, we demonstrated that not only fibrosis but also the intrinsic properties of SCs may be responsible for the poor regeneration of the palate muscles after (surgical) injury. BrHM differ from limb muscles especially in their delayed differentiation. In contrast, the SCs from muscles originating from the different branchial arches are highly similar. SCs from neonatal muscles have a lower fusion efficiency than those from older muscles suggesting a lower regenerative capacity. This study mainly shows the importance of age in muscle regeneration. Although *in vitro* studies not always exactly recapitulate the events occurring *in vivo*, they give insight into the regulation of satellite cell myogenesis. Therefore, further *in vivo* studies into age-dependent differences in SC properties are required to confirm this. This will provide valuable information for future clinical applications in young, growing patients. New therapies should aim to improve muscle regeneration after surgery by preventing fibrosis and promoting SC fusion.

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CHAPTER 7

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

In cleft lip and/or palate (CL/P) patients, surgical closure including repositioning of the muscle sling (levator palatine muscle) fails to restore the function of the soft palate about 7-30%. This results in hypernasal speech that might require additional surgical corrections and speech therapy.[1] In spite of all efforts to accomplish adequate velopharyngeal function, stigmatizing speech aberrations are quite common in these patients.[2]

Skeletal muscles generally possess a remarkable ability to regenerate after injury. The progenitor cells that are responsible for muscle regeneration are the satellite cells (SCs). [3,4] After injury, SCs undergo proliferation, differentiation and fusion to either form new myofibers or to repair damaged ones.[5,6] However, regeneration of the soft palate muscles after surgical repair is suggested to be hampered because of three aspects; (1) their low intrinsic regenerative capacity, (2) the specific muscle properties related to clefting, and (3) the development of fibrosis (chapter 2).

Muscle regeneration can be seriously impaired in different types of congenital degenerative diseases.[7,8] Interestingly, some of the head muscles show a high resistance against degeneration in several of these degenerative diseases such as Duchenne muscular dystrophy.[9] This suggests that head muscles differ from other muscles. Injuries of the head (branchiomeric) muscles heal much slower than similar injuries in limb (somite-derived) muscles.[10] In addition, more fibrous connective is formed during healing compared to limb muscles is formed during healing. SCs from head muscles express a distinct profile of transcription factors including unique factors such as Tcf21.[11] All this indicates that the physiology of SCs from limb and head muscles is different, and that they respond differently to injury.[8] Over the past two decades, skeletal muscle tissue engineering techniques have started to develop.[12,13] In contrast to the extensive research on limb and trunk muscles, studies on head muscles and their regeneration are rare.

Patients with a cleft in the soft palate may benefit of the development of new therapies based on tissue engineering to ameliorate palatal muscle repair. Consequently, this requires both suitable animal models and appropriate *in vitro* culture systems to design and optimize new strategies for soft palate repair. The studies performed in this thesis focus on the biology and regeneration of branchiomeric muscles. These preclinical studies form the basis for the development of new therapies to enhance muscle regeneration in the soft palate and ultimately to improve the quality of life of cleft palate patients and their families.

In the following sections our most interesting findings will be discussed as well as the future perspectives for research in this field.

The animal model

Firstly, we characterized the regeneration of the soft palate muscles in the rat after injury. We developed the first model for soft palate muscle regeneration by making a full-thickness defect in the soft palate to analyze the healing process (chapter 3 and 4). After muscle injury, extensive fibrotic tissue developed in the wound area with little or no formation of new myofibers. This model represents critical aspects of the clinical situation because it allows to study the regeneration of myofibers and the development of fibrosis. However, a drawback is that these animals do not have a congenital cleft and therefore the intrinsic abnormalities of cleft muscles are missing. In the future, a key factor to achieve success will be not only to understand muscle regeneration in the soft palate muscles under normal conditions, but also in cleft muscles. Before translation into the clinic, model systems representing clinical aspects of CL/P are required. The increasing knowledge of specific genes causing CL/P allow the development of genetically engineered mouse models that represent specific abnormalities of CL/P.[14] While the use of these models have limited applications for

postnatal studies due to early perinatal mortality,[15,16] SC isolation from palatal muscles of these animals offer new possibilities for the understanding of SC biology in syndromic CL/P cases. Additionally, the use of larger animal models such as the existing congenital cleft palate model in the goat may be necessary.[17-19]

In summary, we developed a model for compromised muscle regeneration in the soft palate by introducing a full-thickness defect. This model offers promising possibilities for further research. Tissue processes such as collagen deposition and fibrosis can be studied easily. Ultimately novel strategies based on tissue engineering can be developed to improve the outcome of cleft palate surgery. However, in the future, the use of larger cleft palate models may be necessary as they will approach the clinical situation more closely.

The *in vitro* model

In chapter 4, we demonstrated that muscle regeneration in the soft palate starts with the activation, recruitment, and proliferation of SCs from the wound margins. Next, activated SCs migrate into the wound area, proliferate and differentiate into myoblasts. However, recruited differentiating myoblasts failed to fuse and did not form new myofibers within the fibrotic tissue. Was the accumulation of collagen impeding SC differentiation and fusion? Or do head muscle SCs indeed have a lower regenerative capacity as suggested by earlier studies? In the *in vivo* model it is difficult to investigate which specific factors cause the poor muscle regeneration. The next step then was to analyze the differentiation and myotube formation of SCs from branchiomeric head muscles (BrHM). Hence, SCs from BrHM needed to be isolated. However, muscles from the head area in the rat are small and difficult to dissect. For instance, the *levator veli palatini* muscle in the rat is not easily accessible, and its small size makes it difficult to isolate the SCs. In order to obtain enough

SCs for *in vitro* studies, either the cells need to be expanded or larger numbers of animals are required. However, the expansion of primary SCs can seriously limit their proliferative and regenerative capacity.[20] With this in mind, we developed a new method (chapter 5) for the isolation and culture of SCs from head muscles of young rats. After isolation, low numbers of SCs are cultured on extracellular matrix spots of millimeter size to study their proliferation and differentiation. This approach not only avoids the expansion and passaging of SCs but also reduces the number of animals required.

As mentioned before, muscle regeneration in the soft palate of the rat after excisional injury is incomplete. Differentiating SCs within the wound area failed to form new myofibers as large amounts of collagen are deposited. However, also the capacity of BrHM SCs to form myofibers might be impaired. In order to clarify this, in chapter 6, we investigated the differentiation and myotube formation of SCs isolated from muscles originating from the 1st, 2nd and 4th branchial arches. We also compared these cells with limb muscle SCs. Two age groups were included, neonatal (2-weeks old) and young rats (9-weeks old) to evaluate the effects of age.

We confirmed earlier observations that differentiation of BrHM SCs is delayed when compared with muscles from the limb. This delay in differentiation might explain the poor regeneration of BrHM in response to injury. We also demonstrated that SCs from neonatal muscles only form few and small myotubes suggesting age-dependent differences in SCs properties.

There is also evidence that immature growing muscle and full growth muscle behave quite different. For instance, insulin-like growth factor 1 has a hypertrophic effect on skeletal muscle only during muscle growth.[21] Also, the sarcolemma of an actively growing myofiber seems to respond differently to injury. The vesicle-mediated membrane resealing in injured adult muscle is less evident in injured growing muscle.[22,23] In addition, other differences such as calcium regulation, membrane fluidity, and lipid

composition have been reported.[24] Thus, the distinct properties of growing muscle may have large implications for the development of muscle therapies in children.

A limitation of this model is that the cells are grown on solid flat surfaces as a two-dimensional (2D) monolayer. In contrast, a three-dimensional (3D) cell culture offers an artificial environment in which cells are able to grow in all three directions. Cells in a 3D environment compared to 2D environment, show improved viability, morphology, proliferation, and differentiation.[25-27] Therefore, 3D cell culture systems mimics more accurately the *in vivo* situation compared to 2D cell culture systems.[28]

In addition, efforts to gain insight into the cellular mechanisms of human diseases have resulted in the development of cellular disease models. In these models, human cells, including primary cells, established cell lines and more recently, derivates of induced pluripotent stem cells are cultured. In fact, the ability to generate patient-specific induced pluripotent stem cells offers modeling human disease and individualized drug testing.[29,30] Although this field is not yet fully developed, there are already some examples of major success in personalized medicine, recapitulating key features of human disease *in vitro.* For example, a human congenital heart syndrome was recently modeled and further employed to evaluate drugs that may ameliorate the disease phenotype.[30] Fortunately, the rapid advances in this field promise further applications. In the future, 2D or 3D human cultures of cleft palate muscles might reflect both the genetic background and phenotype of CL/P for a specific patient. Hence, these systems could be not only used to mimic disease but also for the testing of regenerative medicine approaches and personalized medicine.[31-33] An important challenge, however, is the possibility to incorporate these personal systems in a routine clinical laboratory setting.[33]

In summary, we developed a rapid and economical method for the isolation and study of SCs derived from head muscles or small

muscle samples. We demonstrated that not only the presence of fibrosis, but also the intrinsic properties of SCs as well as age-depended differences in stem cell properties determine the regeneration of palate muscles after injury. Further studies must be focused on clinical application in growing CL/P patients. However, the transition toward 3D culture systems using specific genetically engineered cells is required. Hopefully in the future, novel humanized models for CL/P will be available.

Regenerative strategies for cleft palate repair

In vivo and *in vitro* results show that both fibrosis and intrinsic characteristics of BrHM SCs contribute to impaired muscle regeneration after injury (chapter 4 and 6). New therapies aimed at preventing fibrosis and promoting myofiber formation are crucial to improve muscle regeneration after soft palate repair. Extracellular matrix components, growth factors, and cytokines may be used to address both problems.

Muscle fibrosis is induced by transforming growth factor-β1 (TGF-β1) and myostatin, both members of the TGF-β super family of growth factors.[34] TGF-β1 plays a significant role in the initiation of fibrosis by inducing myofibroblast differentiation,[34] while myostatin reduces the migration of SCs and also their proliferation and differentiation.[35,36] Therefore, the inhibition of TGF-β1 and myostatin activity may prevent fibrosis and improve muscle healing after injury.[35] Decorin is a member of the small leucine-rich proteoglycan family, which binds TGF-β1 and myostatin thereby reducing their activity.[35,37-39] It may thus prevent fibrosis and enhance myofiber formation.[40,41]

Different phases of adult myogenesis, including SC differentiation and fusion can be regulated through the use of growth factors.[42] For instance, hepatocyte growth factor stimulates satellite cell

differentiation and angiogenesis.[43] However, when growth factors are injected *in vivo*, they rapidly lose their biological activity due to diffusion and/or enzymatic inactivation.[44] Fortunately, in the last years, new biomaterials such as alginate gels have been developed that give a controlled release of growth factors over a period of time.[45,46] These kind of systems may accommodate the long-term presence of growth factors in the injured area. Furthermore, a specific scaffold geometry may guide the proper alignment of differentiating SCs during myofiber formation. This may be achieved with topographical cues such as patterning or microgrooves, which control cytoskeletal alignment, myotube assembly and fusion of differentiating SCs.[47,48]

As mentioned earlier (chapter 2 and 6), the specific muscle properties related to the cleft may also contribute to the poor regeneration of the soft palate muscles after injury. To overcome this, cell-based therapy could be used to increase the number of SCs in the atrophied cleft palate muscles.[49,50] In particular, the masseter muscle could serve as an effective source of SCs. Unfortunately, the delivery of SCs to injured muscle so far has not been very successful.[51] SCs cultured and expanded *in vitro* and then transplanted *in vivo* show a decrease in cell proliferation and in myogenic potential.[51,52] Other limitations, such as poor survival after delivery and tumorigenicity have been reported. Furthermore, neonatal SCs that are not able to form long myofibers might limit their therapeutic efficacy. Overall, it might be more feasible to develop strategies that do not require isolated SCs.

A potent alternative could be '*in situ* guided tissue regeneration' which aims to recruit resident SCs to the injured area.[53,54] The combination of a patterned scaffold with growth factors and cytokines (e.g., stromal cell derived factor–1α) might favor migration and differentiation of resident SCs, and induce myofiber alignment.[34] However, in cases where the availability and quality of SCs is limited cell therapy may still be required.

One important aspect not considered in this thesis is the interaction between SCs and the local immune response during muscle regeneration. Two distinct subpopulations of macrophages are sequentially present in injured muscle tissue. Firstly, pro-inflammatory macrophages secreting pro-inflammatory cytokines such as tumor necrosis factor α and interleukin 1β, phagocytose the necrotic tissue. Secondly, anti-inflammatory macrophages secreting anti-inflammatory cytokines resolve inflammation.[55] Recent research shows that the administration of interleukine-10 to mice with dystrophy deactivates the pro-inflammatory macrophages and reduce pathology. This suggests that modulation of the macrophages population may also improve muscle regeneration.[56-58]

In summary, myofiber formation may be achieved with scaffolds of specific design and surface topography. Additionally, extracellular matrix components, specific growth factors and/or cytokines may limit the development of fibrosis. A better understanding of the immune response in injured muscle tissue may provide additional targets for therapy.

Concluding remarks

It is obvious that new therapies are much needed to improve muscle healing and regeneration after cleft palate repair. However, many questions remain to be answered. For example, the exact contribution of differences in stem cell properties to the poor regeneration of branchiomeric muscles is still unclear. Further research in this aspect will provide valuable information for clinical application in growing patients.

A key factor to achieve success will not only be to understand muscle regeneration in the soft palate muscles under normal conditions, but also in cleft muscles. Hence, later studies on SC from clefted muscles by using animals with a cleft or transgenic

models are warranted. Another important step before translation into the clinic will be the use of larger animal models to test potential new therapies.

In this research project, we developed the first animal model for muscle regeneration in the soft palate, which can be used to investigate and develop new treatment strategies for soft cleft palate repair. Next, an innovative method for the isolation and culture of SCs was established that allows the study of factors that regulate SC differentiation and myotube formation. The results from this thesis indicate that not only fibrosis, but also the intrinsic characteristics of SCs from BrHM contribute to defective regeneration after injury. This research project is the first step into the development of novel therapies to improve cleft palate repair and the quality of life of patients with CL/P.

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CHAPTER 8

SUMMARY / SAMENVATTING

In chapter 1, the background and rationale of this study is explained. Children with a cleft in the soft palate have difficulties with speech, swallowing and sucking. These patients are unable to separate the nasal from the oral cavity leading to air loss during speech. Although surgical repair ameliorates soft palate function by joining the clefted muscles of the soft palate, optimal function is in some cases not achieved. Adjuvant therapies based on tissue engineering might be suitable to improve muscle function after surgery Therefore, the aim of this research project was to gain knowledge in the understanding of the biology and regeneration of branchiomeric head muscles.

The regeneration of muscles in the soft palate after surgery is hampered because of (1) their low intrinsic regenerative capacity, (2) the muscle properties related to clefting, and (3) the development of fibrosis. Adjuvant strategies based on tissue engineering may improve the outcome after surgery by approaching these specific issues. In chapter 2 an overview of myogenesis in the non-cleft and cleft palate, the characteristics of soft palate muscles and the process of muscle regeneration is presented. In addition, novel therapeutic strategies based on tissue engineering to improve soft palate function after surgical repair are proposed.

In chapter 3, a new *in vivo* model for the study of muscle regeneration in the soft palate of rats is established. This model is suitable to study muscle regeneration in the soft palate after surgical injury, and allows the development of novel adjuvant strategies to promote muscle regeneration. This offers new perspectives for the treatment of patients with cleft lip and/or palate, and for various other conditions in which the regeneration of head muscles is compromised.

In chapter 4, we study the long–term regeneration of the soft palate muscles after excisional injury. Muscle regeneration begins with the activation, recruitment and proliferation of satellite cells from the wound margins early in wound healing. Howevepr,

differentiated myoblasts within the wound fail to form new myofibers and large amounts of collagen are deposited in the wounded area. Fibrosis as well as defective muscle regeneration can hamper the functional recovery of the soft palate muscles after cleft palate repair.

In order to determine whether the intrinsic properties of satellite cells from branchiomeric head muscles also contribute to impaired muscle regeneration in the soft palate, *in vitro* culture systems are required. Therefore, in chapter 5 a new protocol for the isolation, culture and differentiation of satellite cells from head muscles is presented. After isolation, low numbers of satellite cells are cultured on Matrigel spots of millimeter size to study their differentiation. This approach avoids the expansion and passaging of cells. In conclusion, this protocol offers new possibilities to study satellite cells derived from branchiomeric head muscles or other small muscles or muscle samples.

In chapter 6, the fiber type distribution and the expression of satellite cell markers were studied in *ex vivo* muscle tissue. Next, the *in vitro* differentiation and myotube formation of satellite cells isolated from representative muscles originating from the 1st, 2nd and 4th branchial arches were investigated. This was done with satellite cells from neonatal (2-week-old) and young (9-week-old) rats to study the effects of age. In this study, we demonstrate that not only fibrosis but also the intrinsic properties of satellite cells may be responsible for the poor regeneration of the palate muscles after (surgical) injury. Moreover neonatal satellite cells appear to differentiate less than young satellite cells.

In chapter 7, the results of the subsequent studies are discussed. We developed a model for compromised muscle regeneration in the soft palate by introducing a full-thickness defect. This model offers promising possibilities for further research. However, in the future, the use of larger cleft palate models may be necessary as they will approach the clinical situation more closely. We also developed a rapid and economical method for the isolation and

study of satellite cells derived from head muscles or small muscle samples. We confirmed earlier observations that differentiation of satellite cells from branchiomeric head muscles is delayed when compared with muscles from the limb. This delay in differentiation might explain the poor regeneration of branchiomeric head muscles in response to injury. We also demonstrated that age-depended differences in stem cell properties can determine the regeneration of palate muscles after injury.

Further research on age-dependent differences in stem cell properties will provide valuable information for clinical application in growing patients. Also, additional studies on satellite cells from clefted muscles from transgenic animals with a cleft are warranted. In the future, potential new therapies will have to be tested in larger animal models. This research project is the first step into the development of novel therapies to improve cleft palate repair and the quality of life of patients with a cleft in the palate.

In hoofdstuk 1, wordt de achtergrond en het belang van deze studie beschreven. Kinderen met een aangeboren gespleten gehemelte (schisis) hebben moeite met spreken, slikken en zuigen. Deze patiënten zijn niet in staat de neus– en mondholte van elkaar te scheiden waardoor luchtverlies optreedt tijdens het spreken. Ondanks succesvolle sluiting en correctie van de spieren in het zachte gehemelte wordt een optimale functie in sommige gevallen niet behaald. Adjuvante therapie gebaseerd op tissue engineering kan geschikt zijn om de spierfunctie te verbeteren na de operatie. Het doel van dit onderzoeksproject is om de biologische processen tijdens de regeneratie van spieren van het hoofd beter te begrijpen.

De regeneratie van de spieren in het zachte gehemelte na de operatie wordt belemmerd door (1) de lage intrinsieke regeneratieve capaciteit, (2) de spiereigenschappen ten gevolge van de schisis en (3) de ontwikkeling van fibrose. Adjuvante strategieën gebaseerd op tissue engineering kunnen de uitkomst na operatie verbeteren door het aanpakken van deze specifieke problemen. In hoofdstuk 2 wordt een overzicht van myogenese in het niet-gespleten en het gespleten gehemelte gegeven. De kenmerken de spieren in het zachte gehemeltes en het proces van spierregeneratie worden gepresenteerd. Bovendien worden nieuwe therapeutische strategieën gebaseerd op tissue engineering voorgesteld om de functie van het zachte gehemelte na chirurgie te verbeteren.

In hoofdstuk 3 wordt een nieuw *in vivo* model voor de studie van spierregeneratie in het zachte gehemelte van ratten gepresenteerd. Dit model is geschikt om spierregeneratie na chirurgisch letsel in het zachte gehemelte te bestuderen, en maakt de ontwikkeling van nieuwe adjuvante strategieën voor spierregeneratie mogelijk. Dit biedt nieuwe perspectieven voor de behandeling van patiënten met een aangeboren gespleten lip en/of gehemelte, en voor andere aandoeningen waarbij de regeneratie van hoofdspieren is vestoord.

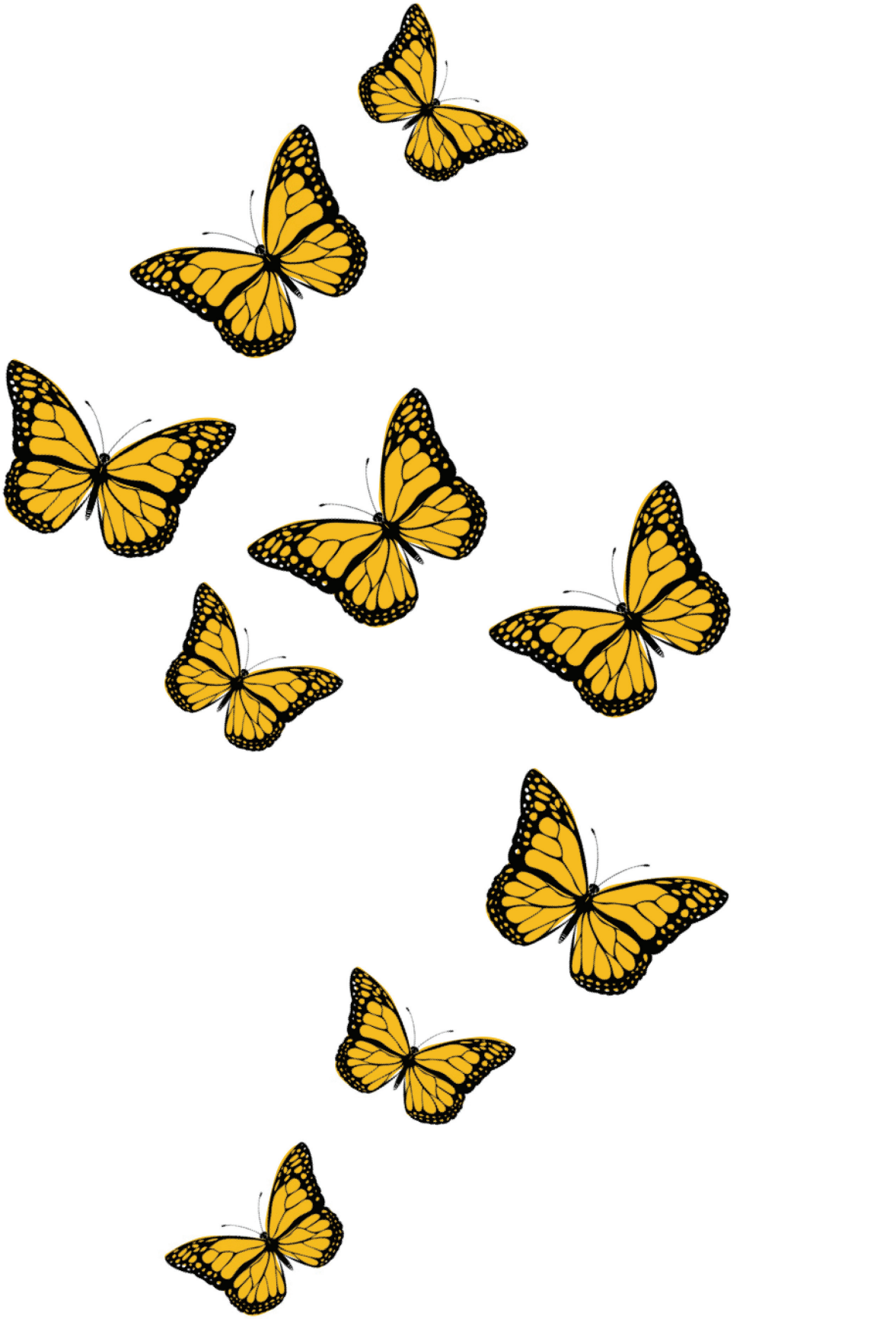
In hoofdstuk 4 wordt de lange termijn regeneratie van de spieren in het zachte gehemelte bestudeerd na verwonding met behulp van *in vivo* model. Na verwonding begint spierregeneratie met de activatie, rekrutering, proliferatie en differentiatie van satellietcellen vanuit de wondranden. Echter, gedifferentieerde myoblasten in de wond blijken geen nieuwe spiervezels te kunnen vormen en grote hoeveelheden collageen worden afgezet in het wondgebied. Fibrose en onvolledige spierregeneratie belemmert het functioneel herstel van de spieren in het zachte gehemelte na chirurgie.

Om te bepalen of naast fibrose de intrinsieke eigenschappen van satellietcellen van de spieren van het hoofd bijdragen aan verminderde spierregeneratie zijn er *in vitro* kweeksystemen vereist. Daarom wordt in hoofdstuk 5 een nieuw protocol voor de isolatie, kweek, en differentiatie van satellietcellen van de spieren van het hoofd ontwikkeld en beschreven. Na isolatie worden kleine aantallen satellietcellen gekweekt op Matrigel spots van enkele millimeters om hun differentiatie te bestuderen. Deze benadering vermijdt de expansie en het doorzetten van cellen. Kortom: dit protocol biedt nieuwe mogelijkheden om satellietcellen afkomstig van de spieren van het hoofd en ook van andere kleine spieren of spiermonsters te bestuderen.

In hoofdstuk 6, wordt het spiervezeltype en de expressie van satellietcellen-markers onderzocht in *ex vivo* spierweefsel. Daarnaast worden de *in vitro* differentiatie en myotube vorming van geïsoleerde satellietcellen uit representatieve spieren afkomstig van de 1^{e}, 2^{e}, en 4^{e} kieuwbogen in het hoofd onderzocht. Dit is gedaan met satellietcellen van neonatale (2 weken oude) en jonge (9 weken oude) ratten om het effect van de leeftijd te bestuderen. In deze studie hebben we aangetoond dat niet alleen fibrose maar ook de intrinsieke eigenschappen van satellietcellen bijdragen aan de slechte regeneratie van de spieren na chirurgisch letsel. Bovendien blijken satellietcellen in neonatale spieren minder goed te differentiëren dan satellietcellen uit jonge spieren.

In hoofdstuk 7 wordt de betekenis van onze resultaten voor toekomstige studies besproken. We hebben een model ontwikkeld voor verstoorde spierregeneratie in het zachte gehemelte door het maken van een excisie wond. Dit model biedt veel mogelijkheden voor verder onderzoek. In de toekomst is echter ook onderzoek nodig in grotere dieren, omdat dit de klinische situatie beter benadert. We hebben ook een nieuwe, snelle en economische methode ontwikkeld voor de isolatie en studie van satellietcellen afkomstig van de spieren van het hoofd of kleine spier monsters. We bevestigen eerdere observaties dat de differentiatie van de spieren van het hoofd trager is dan die van de spieren van de ledematen. Deze vertraging in differentiatie zou kunnen bijdragen aan de gebrekkige regeneratie van de spieren van het hoofd na letsel. We hebben ook aangetoond dat de leeftijdsafhankelijke verschillen in satelliet celeigenschappen de regeneratie van gehemeltespieren beïnvloeden.

Verder onderzoek naar leeftijdsafhankelijke verschillen in stamcel eigenschappen zal waardevolle informatie opleveren voor klinische toepassing in groeiende schisis patiënten. Ook aanvullende studies aan satellietcellen van spieren van transgene dieren met een gespleten gehemelte zijn wenselijk. In de toekomst zullen potentiële nieuwe therapieën moeten worden getest in grotere diermodellen. Dit onderzoek is de eerste stap naar de ontwikkeling van nieuwe therapieën om de functie van het zachte gehemelte en de kwaliteit van leven van patiënten met een schisis te verbeteren.



Like Mauricio Babilonia[1] who is constantly being swarmed by yellow butterflies, I am also being immersed by the lovely memories of my father. Those memories even followed me to The Netherlands and therefore this thesis is all about him… about everything that is magical and realistic… about unusual and extraordinary events… and now I am at the end of this adventure, which of course I could not have finished without the help of others. Therefore, I would like to thank all people who accompanied me during this time and made it a delightful journey.

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[1] Character of **"One Hundred Years of Solitude"** (Spanish: Cien años de soledad) by Colombian Nobel Prize author Gabriel García Márquez.

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Paola Liliana Carvajal Monroy was born in Bogotá, Colombia on 18 February 1976. Her parents are †Aldemar Carvajal Arias, an architect, and Julia Ines Monroy Fernandez, a dentist. Paola's very first dream was to become a writer/journalist. At the age of 10 years she won a prize for her first short story called **'a very strange family',** and in 1990 she was the founder and editor in chief of a newspaper for the scouting association in Bogotá.

During several years, Paola worked passionately on human rights & family health. In 1997, she received the Doctor of Dental Surgery (D.D.S.) degree from the National University of Colombia with the thesis **'Level of knowledge and care of victims of child abuse'**. In the same year she also wrote her first theater play called **"the white house"**, a reflection on the violence and civil war in Colombia. After her graduation, she performed her social service as a dentist in Suarez, Cauca; a small town in the south of Colombia. In 2003, she successfully finished the postgraduate training as Maxillofacial Surgeon from the Pontifical Xavierian University in Bogotá with the thesis **'Characteristics and anatomical variations of the infraorbital foramen, canal, and groove in the Colombian population'**.

In the last year of her surgical training, Paola was selected for a surgical fellowship under supervision of Prof. Dr. Riediger at the University Hospital Maastricht in The Netherlands. During this experience, she not only greatly enjoyed working in a multidisciplinary scientific team, but also decided to move definitely to 'De Lage Landen' because of love. The first years in the Netherlands she combined Dutch Language lessons with training courses at the Academy (of Education) Fine Arts and Design in Maastricht, and the Gerrit Rietveld Academy in Amsterdam where she studied Fine Arts for 1 year.

Although her degrees were not recognized by the European Union, Paola was very keen to start all over again with dentistry and she obtained the DDS degree *cum laude* from ACTA, Amsterdam in 2009. She continued an orthodontics training at the Radboud University Medical Centre (Radboudumc), finishing in 2013. During her training, she had the opportunity to participate in the multidisciplinary Cleft Palate-Craniofacial Unit. The intimate contact with cleft patients and their families inspired her to propose the present Ph.D. Project in collaboration with Johannes Von den Hoff and Frank Wagener. In 2012, Paola obtained a Mosaic grant (017.009.009) from the Netherlands Organization for Scientific Research (NWO) and other small grants for open access publication. In 2013, she got a start grant (S-13-167C) for young investigators from the AO Foundation and from the Osteology

foundation (12-008). In 2014, she won the best poster award from the European Orthodontic Society in Warsaw, Poland. In 2016, she was invited as speaker at the Birth Defects Research Centre from the University College London; Institute of Child Health, United Kingdom. Additionally, in the same year, Paola won the prize for the best publication from the Dutch Society of Medical and Dental Interaction (VMTI) with her article presented in chapter 4.

Nowadays, Paola combines her work as an orthodontist at the Craniofacial Team in the Erasmus Medical Center, Sophia's Children's Hospital, Rotterdam, in the Department of Oral & Maxillofacial Surgery, Special Dental Care, and Orthodontics (head: Prof. Dr. E.B Wolvius), with a private practice together with Stephen Tjoa. She will continue working in cleft palate research as a postdoctoral fellow at the Radboudumc in Nijmegen.

Publications

1. Carvajal Monroy PL, Grefte S, Kuijpers-Jagtman AM, Helmich MP, Wagener FA, Von den Hoff JW. Neonatal satellite cells form small myotubes *in vitro*. J Dent Res (submitted)

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